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(54) Title: INTRANASAL VACCINATION AGAINST GASTROINTESTINAL DISEASE

(57) Abstract

The invention features intranasal immunization methods for inducing immune responses in distal mucosal sites, e.g., the gastrointestinal or genitourinary tracts. The methods of the invention may be used to induce protective and/or therapeutic immune responses against pathogens (e.g., bacteria of the genus *Clostridium*, e.g., *C. difficile*) which infect these distal sites. Also included in the invention are vaccination methods in which combinations of mucosal (e.g., oral or intranasal) and parenteral (e.g., subcutaneous or intraperitoneal) routes of administration are used.

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INTRANASAL VACCINATION AGAINST GASTROINTESTINAL DISEASE

Background of the Invention

This invention relates to intranasal vaccination methods for preventing and/or treating gastrointestinal disease.

Clostridium difficile is a gram-positive, spore-forming, toxigenic bacterium that causes antibiotic-associated diarrhea which can progress into severe and sometimes fatal colitis. Upon disruption of the normal intestinal flora by, *e.g.*, antibiotic or anti-neoplastic therapy, *C. difficile* may become established in the colon where it produces two high molecular weight toxins, Toxin A and Toxin B. Both of these polypeptides are cytotoxins, but Toxin B is greater than 1000-fold more potent than Toxin A. Toxin A is also an enterotoxin, as it causes accumulation of fluid in ligated animal intestinal loops.

Summary of the Invention

We have shown that intranasal, and combined mucosal (*e.g.*, oral or intranasal) and systemic (*e.g.*, subcutaneous or intraperitoneal), vaccination regimens, even in the absence of an adjuvant, are effective in inducing mucosal immune responses at distal mucosal sites (*e.g.*, the gastrointestinal and/or genitourinary tracts). Vaccination of hamsters with *C. difficile* toxins A or B (or toxoids) using either of these methods gives rise to protection of these animals from subsequent *C. difficile* challenge.

Accordingly, the invention features a method of inducing a distal mucosal immune response (*i.e.*, a mucosal immune response outside of the upper respiratory tract, *e.g.*, in the gastrointestinal and/or genitourinary tracts) to a gastrointestinal or genitourinary tract

pathogen in a mammal. In this method, a non-replicatable polypeptide antigen which is dissolved in a pharmaceutically acceptable diluent, and which is capable of inducing the distal immune response to the pathogen, is administered intranasally to the mammal.

The invention also features a method of inducing a distal mucosal immune response to a pathogen in a mammal involving: (1) administering an antigen capable of inducing the distal immune response to a mucosal surface of the mammal, and (2) parenterally administering the antigen to the mammal. Any order of combined mucosal and parenteral administration is included in the invention. For example, mucosal (*e.g.*, intranasal, oral, ocular, gastric, rectal, vaginal, gastrointestinal, or urinary tract) administration may precede parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, or intramuscular) administration, or parenteral administration may precede mucosal administration. As an example, three weekly doses may be administered mucosally (*e.g.*, intranasally) and, on the fourth week, combined mucosal (*e.g.*, intranasal) and parenteral (*e.g.*, intraperitoneal) administration may carried out.

Pathogens to which mucosal immune responses may be induced in the methods of the invention, and from which the antigens (*e.g.*, non-replicatable polypeptide antigens) may be derived, include, but are not limited to gastrointestinal pathogens such as *Helicobacters* (*e.g.*, *H. pylori*, *H. felis*, and *H. heilmanii*), *Campylobacters* (*e.g.*, *C. jejuni*), pathogens which cause diarrhea and colitis (*e.g.*, *Clostridia* (*e.g.*, *C. difficile*, *C. novyi*, and *C. sordellii*), enterotoxigenic *E. coli*, Shigella, *Vibrio cholerae*, and *Salmonella typhi*), and genitourinary tract pathogens (*e.g.*, human immunodeficiency virus, herpes simplex viruses, papilloma viruses, *Treponema pallidum*, *Chlamydia*, and *Neisseria gonorrhoeae*).

Specific examples of antigens (*e.g.*, non-replicatable polypeptide antigens) that may be used in the methods of the invention include, but are not limited to, bacterial toxins. For example, toxins from *Clostridia* (*e.g.*, *C. difficile*, *C. novyi*, and *C. sordellii*), such as *C. difficile* Toxin A and/or B Toxoid, *C. novyi* α-toxin (Bette *et al.*, Toxicon 29(7):877-887, 1991), *C. sordellii* lethal toxin (Bette *et al.*, *supra*), and immunogenic fragments and derivatives thereof, may be used. The antigens used in the methods of the invention may be obtained by standard methods known in the art, *e.g.*, purification from a culture of the pathogen from which it is derived, recombinant DNA methods, and chemical synthetic methods.

The invention may employ *Clostridium* (*e.g.*, *C. difficile*) toxoids as vaccine antigens. A toxoid is a toxin (or mixture of toxins, *e.g.*, *C. difficile* Toxin A and Toxin B) that has been treated so as to decrease the toxic properties of the toxin(s), but to retain antigenicity. Toxoids included in the invention are made using standard methods including, but not limited to, chemical (*e.g.*, formaldehyde or glutaraldehyde) treatment, protease cleavage, and recombinant methods (*e.g.*, by making fragments or mutations (*e.g.*, point mutations) of the toxin(s)).

The method of the invention may be carried out in order to prevent or decrease the chances of a future infection by a pathogen (*i.e.*, to induce a protective immune response) and/or to treat an ongoing infection (*i.e.*, to induce a therapeutic immune response). In the case of intestinal pathogens, for example, the method of the invention may be used to treat a mammal that is at risk of developing, but does not have, diarrhea caused by the pathogen (*e.g.*, *C. difficile*), or a mammal that has diarrhea caused by the pathogen. Mammals which may be

treated according to the method of the invention include, *e.g.*, humans, cows, horses, pigs, dogs, cats, sheep, and goats.

An advantage of the methods of the invention is that, for at least some antigens (*e.g.*, *C. difficile* toxins and toxoids), mucosal adjuvants are not required for induction of an immune response (*e.g.*, a protective immune response).

Other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments thereof, and from the claims.

Detailed Description

The drawings are first described.

Drawings

Fig. 1 is a graph showing the levels of protection against *C. difficile* disease in hamsters immunized with *C. difficile* antigens by the indicated routes. The levels of protection from systemic (death) and intestinal (diarrhea) disease after clindamycin challenge are shown. (See Table 1 for a description of the immunization routes.)

Fig. 2 is a graph showing the mean (+SE) antibody titers to *C. difficile* Toxin A, Toxin B, and whole cell antigens in sera from hamsters after 3 doses of vaccine administered by the routes indicated, as determined by ELISA. (See Table 1 for a description of the routes of immunization.) Sera from hamsters after 3 doses of vaccine were assayed for specific IgG; the titer was defined as the maximum dilution with an absorbance of >0.3. Each bar represents the mean (+SE) of five animals.

Fig. 3 is a graph showing the biological activity of sera from hamsters administered 3 doses of vaccine by

the indicated routes. Sera were tested for inhibition of cytotoxin A or cytotoxin B activity in IMR-90 cells, and for agglutination of *C. difficile* cells; titers were defined as the maximal dilution with biological activity. Each bar represents the mean (+SE) of five animals. (See Table 1 for a description of the routes of immunization.)

Fig. 4 is a graph showing the long term antibody response in i.n.i.p. and s.c. immunized hamsters. Comparisons of the responses before clindamycin challenge (i.n.i.p.-I and s.c.-I) and 140 days after clindamycin challenge (i.n.i.p.-II and s.c.-II) are shown. Sera were tested by ELISA against Toxin A, Toxin B, and whole cell antigens, and the titers were expressed as the maximal dilution with absorbance >0.3; each bar represents the mean (+SE) of five animals.

Fig. 5 is a graph showing the long term antibody response in i.n.i.p. and s.c. immunized hamsters. Comparisons of the responses before clindamycin challenge (i.n.i.p.-I and s.c.-I) and 140 days after clindamycin challenge (i.n.i.p.-II and s.c.-II) are shown. Sera was tested for inhibition of cytotoxins in IMR-90 cells and for agglutination of *C. difficile* cells; the titer was the maximal dilution of serum with biological activity. Each bar represents the mean (+SE) of five animals.

Figs. 6A-6B are graphs showing the anti-Toxin A (Fig. 6A) and anti-Toxin B (Fig. 6B) IgA responses in serum, feces, saliva, and vaginal secretions of mice after intranasal immunization with toxoid, in the presence or absence of CT.

Figs. 7A-7C are graphs showing the serum anti-Toxin B cytotoxicity after intranasal immunization of mice with toxoid (Fig. 7A), the serum anti-Toxin A cytotoxicity after intranasal immunization with toxoid (Fig. 7B), and the salivary and vaginal secretion anti-

Toxin A cytotoxicity after intranasal immunization with toxoid (Fig. 7C).

Fig. 8 is a graph showing the level of passive protection of ligated small intestinal loops of rats from Toxin A using sera from mice immunized intranasally with toxoid.

Fig. 9 is a graph showing the percent survival of mice intranasally immunized with toxoid after lethal challenge with Toxin A or Toxin B.

Fig. 10 is a graph showing the level of Toxin A enterotoxicity in ligated intestinal loops of mice after intranasal immunization of toxoid.

Figs. 11A-11B are graphs showing the Toxin A-specific systemic (Fig. 11A) and mucosal (Fig. 11B) IgA responses after immunization with GST-ARU by the indicated routes. (See Table 5 for a description of the routes of immunization.)

Figs. 12A-12B are graphs showing the levels of Toxin A cytotoxicity inhibition of sera taken 40 days after immunization with GST-ARU. (See Table 5 for a description of the routes of immunization.)

Figs. 13A-13B are graphs showing the levels of passive inhibition of Toxin A enterotoxicity in rat intestinal loops with immune sera from GST-ARU immunized mice. (See Table 5 for a description of the routes of immunization.)

Fig. 14 is a graph showing the percent survival from lethal Toxin A challenge after immunization with recombinant Toxin A repeats (ARU). (See Table 5 for a description of the routes of immunization.)

Fig. 15 is a graph showing the levels of protection from enterotoxicity of Toxin A in ligated mouse intestinal loops after immunization with GST-ARU. (See Table 5 for a description of the routes of immunization.)

Intranasal and Combined Mucosal-Systemic Vaccination Methods for Inducing Mucosal Immune Responses at Distal Sites

We have shown that intranasal, or combined mucosal and systemic, administration regimens give rise to mucosal immune responses in the gastrointestinal and genitourinary tracts.

The methods of the invention may be used to induce protective and/or therapeutic immune responses to gastrointestinal pathogens including, but not limited to, *Helicobacters* (*e.g.*, *H. pylori*, *H. felis*, and *H. heilmanii*) *Campylobacters* (*e.g.*, *C. jejuni*), and pathogens which cause diarrhea and colitis, *e.g.*, *Clostridia*, enterotoxigenic *E. coli*, Shigella, *Vibrio cholerae*, and *Salmonella typhi*; or genitourinary tract pathogens (*e.g.*, human immunodeficiency virus, herpes simplex viruses, papilloma viruses, *Treponema pallidum*, *Chlamydia*, and *Neisseria gonorrhoeae*). Appropriate vaccine antigens (*e.g.*, polypeptide antigens), corresponding to the pathogen which causes the condition desired to be prevented and/or treated using the method of the invention, are readily selected by one skilled in the art. The methods of the invention are described, as follows, referring to antigens from *C. difficile* (*e.g.*, toxins or toxoids) as specific examples of vaccine antigens which may be used in the methods of the invention.

Use of *C. difficile* toxins and toxoids as vaccines

C. difficile toxin polypeptides which may be used in the methods and compositions of the invention can be prepared using any of several standard methods. For example, the toxins (*e.g.*, Toxin A and/or Toxin B) can be purified from *C. difficile* culture filtrates (see, *e.g.*,

Kim *et al.*, Infection and Immunity 55:2984-2992, 1987; and see Example I, below).

C. difficile toxin polypeptides can also be produced using standard recombinant DNA methods (see, *e.g.*, Ausubel *et al.*, Eds., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 1994). In these methods, a suitable host cell is transformed with an appropriate expression vector containing all or part of a toxin-encoding nucleic acid fragment (see Dove *et al.*, Infection and Immunity 58:480-488, 1990, and Barroso *et al.*, Nucleic Acids Research 18:4004, 1990, for the nucleotide and deduced amino acid sequences of *C. Difficile* Toxin A, and the nucleotide sequence of Toxin B, respectively). Any of a variety of expression systems can be used to produce the recombinant toxins. For example, the toxin polypeptides can be produced in a prokaryotic host (*e.g.*, *E. coli*) or in a eukaryotic host (*e.g.*, yeast cells (*e.g.*, *Saccharomyces cerevisiae*), mammalian cells (*e.g.*, COS1, NIH3T3, or JEG3 cells), or arthropod cells (*e.g.*, *Spodoptera frugiperda* (SF9) cells)). Such cells are available from a number of different sources known to those skilled in the art, *e.g.*, the American Type Culture Collection, Rockville, MD (also see, *e.g.*, Ausubel *et al.*, *supra*). The transfection/transformation method used, and the choice of expression vector, will depend on the host system selected, as is described by, *e.g.*, Ausubel *et al.*, *supra*. Expression vectors (*e.g.*, plasmid or viral vectors) can be chosen from, *e.g.*, those described in *Cloning Vectors: A Laboratory Manual* (Pouwels *et al.*, 1985, Supp. 1987; also see, *e.g.*, Ausubel *et al.*, *supra*).

C. difficile toxin polypeptides, particularly short fragments, can also be produced by chemical synthesis, *e.g.*, by the method described in *Solid Phase Peptide Synthesis*, 1984, 2nd ed., Stewart and Young,

Eds., Pierce Chemical Co., Rockford, IL, and by standard *in vitro* translation methods.

Toxoids of *C. difficile* toxins can also be used in the methods of the invention. A toxoid is a toxin that has been treated so that the toxicity of the toxin is eliminated or reduced, but the antigenicity is maintained. Toxoids may be prepared using standard methods, for example, by chemical (*e.g.*, glutaraldehyde or formaldehyde) treatment (see, *e.g.*, Libby *et al.*, Infection and Immunity 36:822-829, 1982). Toxoids may also be prepared by making mutations in the genes encoding the toxins and expressing the mutated genes in an expression system, as is described above. Regions in Toxin A and/or Toxin B that can be mutated include, *e.g.*, the conserved cysteine residues, the nucleotide binding region, the internal hydrophobic region, and/or the carboxyl-terminal repeat regions. Specific examples of such mutations in *C. difficile* toxins which can be used in the invention are described by, *e.g.*, Barroso *et al.*, Microbial Pathogenesis 16:297-303, 1994.

Other methods of producing toxoids that can be used in the invention include chemical modification of amino acids which are critical for toxicity, but are not related to antigenicity. For example, reagents which specifically modify SH-containing amino acids, lysine, tyrosine, tryptophan, or histidine residues are known in the art (see, *e.g.*, Cohen *et al.*, Ann. Rev. Biochem. 37:683-695, 1968). In addition, azido-linked substrate analogs, such as UDP-glucose, which can be covalently linked to toxin active sites by ultraviolet irradiation, can be used to produce toxoids.

In addition to native, full length, *C. difficile* toxins, polypeptide fragments of toxins, or toxins (or polypeptide fragments of toxins) containing mutations (which may or may not be toxoids) can be used in the

invention, provided that antigenicity is retained. For examples of fragments of *C. difficile* toxins, see, *e.g.*, Price *et al.*, Current Microbiology 16:55-60, 1987; Lyerly *et al.*, Current Microbiology 21:29-32, 1990; and Frey *et al.*, Infection and Immunity 60:2488-2492, 1992. Genes encoding fragments of *C. difficile* toxins, and/or toxins containing mutations, are made using standard methods (see, *e.g.*, Ausubel *et al.*, *supra*). Fragments, derivatives, and toxoids included in the invention can be screened for antigenicity using standard methods in the art, *e.g.*, by measuring induction of a mucosal immune response (see below), induction of protective immunity (see below), or induction of a therapeutic immune response.

Although not required, adjuvants may be administered with the vaccines in the methods of the invention. Any of a number of adjuvants that are known to one skilled in the art may be used. For example, a cholera toxin (CT), the heat-labile enterotoxin of *Escherichia coli* (LT), or fragments or derivatives thereof having adjuvant activity, can be used for mucosal administration. An adjuvant such as RIBI (ImmunoChem, Hamilton, MT) or aluminum hydroxide can be used for parenteral administration.

Fusion proteins containing a *C. difficile* toxin (or a fragment or derivative thereof) fused to, *e.g.*, an adjuvant (*e.g.*, CT, LT, or a fragment or derivative thereof having adjuvant activity), are also included in the invention, and can be prepared using standard methods (see, *e.g.*, Ausubel *et al.*, *supra*). In addition, the vaccines of the invention can be covalently coupled or cross-linked to adjuvants. Methods for covalently coupling and chemically cross-linking adjuvants to antigens are described by, *e.g.*, Cryz *et al.*, Vaccine 13:67-71, 1994; Liang *et al.*, J. Immunology 141:1495-

1501, 1988; and Czerkinsky *et al.*, Infection and Immunity 57:1072-1077, 1989.

As is mentioned above, vaccine compositions (with or without adjuvants) are administered intranasally according to the methods of the invention. Combined modes of administration may also be used, *e.g.*, the first dose of the vaccine can be administered to a mucosal (*e.g.*, intranasal or oral) surface, and booster immunizations can be administered parenterally (*e.g.*, intraperitoneally or subcutaneously); this combination gives unexpectedly good results. For example, a parenteral booster immunization may be given one week after the first, mucosal administration.

The amount of vaccine administered depends on the particular vaccine antigen, whether an adjuvant is co-administered with the vaccine antigen, the mode and frequency of administration, and the desired effect (*e.g.*, protection and/or treatment), as can be determined by one skilled in the art. In general, the vaccine antigens of the invention are administered in amounts ranging between, *e.g.*, 1 μg and 100 mg. If adjuvants are administered with the vaccines, amounts ranging between, *e.g.*, 1 ng and 1 mg can be used. Administration is repeated as necessary, as can be determined by one skilled in the art. For example, a priming dose can be followed by 3 booster doses at weekly intervals. Vaccines may be administered in any pharmaceutically acceptable carrier or diluent (*e.g.*, water, a saline solution (*e.g.*, phosphate-buffered saline), or a bicarbonate solution (*e.g.*, 0.2 M $NaHCO_3$)). The carriers and diluents used in the invention are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use in

pharmaceutical formulations, are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field, and in the USP/NF.

The following examples are meant to illustrate, but not to limit, the methods of the invention. Modifications of the conditions and parameters set forth below that are apparent to one skilled in the art are included in the invention.

EXAMPLES

Two model systems, the mouse and the hamster, were used to evaluate the vaccination methods of the invention. Because hamsters are susceptible to antibiotic-associated diarrhea which is similar to that of humans, the hamster model was used to directly evaluate the protective efficacy of vaccination against *C. difficile* disease. *C. difficile* infection of hamsters causes severe hemorrhagic cecitis, which is reminiscent of the colitis observed in the human disease state. In addition, oral or systemic administration to a hamster of a single dose of clindamycin, in combination with *C. difficile*, results in severe diarrhea, which ultimately leads to death of the animal.

Using a variety of assays, the hamster model may also be used to monitor the immune response induced by the vaccination methods of the invention. For example, serum and mucosal samples from immunized hamsters can be used to measure inhibition of *in vitro* cytotoxicity. In addition, ligated intestinal loops of immunized hamsters can be used to evaluate the inhibition of the enterotoxic activity of Toxin A induced by vaccination. Further, colonization of hamsters with *C. difficile* can be monitored by fecal culture, or the presence of Toxin A and/or Toxin B in hamster feces can be determined by ELISA and/or cytotoxicity analysis.

Features of the mouse model are advantageous in evaluating the immune responses induced by the vaccination methods of the invention. Specifically, monoclonal antibodies which recognize mouse IgA are commercially available, and thus facilitate evaluation of the mouse mucosal immune response. In contrast, such reagents are not available for evaluating the hamster mucosal immune response. An additional advantage of the mouse model is that methods for sampling mouse mucosal surfaces have been developed which allow mucosal responses to various immunization regimens to be mapped.

Once the immunogenicity of a vaccine candidate is established by, *e.g.*, ELISA analysis, mouse serum samples can be used to investigate properties of the antibodies which are likely to be associated with effective vaccines. For example, serum from immunized mice can be analyzed for its ability (1) to inhibit *in vitro* cytotoxicity of Toxin A and/or Toxin B, or (2) to inhibit the enterotoxicity of Toxin A using ligated intestinal loops of mice or rats challenged with Toxin A. Immunized mice may also be challenged orally, or in their ligated intestinal loops, to determine protection from death or fluid accumulation due to Toxin A enterotoxicity. Finally, immunized mice may be challenged with toxins systemically with doses known to be lethal.

Example I. Immunization of Hamsters with Vaccine Compositions Containing *C. difficile* Toxins

The following methods were used to analyze the efficacy of the immunization methods of the invention in the hamster model system.

Preparation of a *C. difficile* Toxoid Vaccine

C. difficile culture filtrate was prepared and inactivated as described by Libby, *et al.* (Infection and

Immunity 36:822-829, 1982). Briefly, *C. difficile* VPI strain 10463 (ATCC accession number 43255) was grown for 3 days in dialysis flasks, centrifuged, and filter sterilized. One ml of formaldehyde was added to 100 ml of the culture filtrate, and the mixture was incubated at 37°C for 1 hour. The culture filtrate had a concentration of approximately 50 μg/ml of Toxin A, as determined by ELISA (Lyerly, *et al.*, Infection and Immunity 47:349-352, 1985), and a cytotoxic titer of 10^6 for Toxin B, as determined by a cell culture cytotoxicity assay (Ehrich, *et al.*, Infection and Immunity 28:1041-1043, 1980). The toxoid was washed with 3 volumes of phosphate buffered saline (PBS), pH 7.4, by ultrafiltration through a 30 kD membrane in a 500 ml cell concentrator (Amicon, Beverly, MA). The toxoid was concentrated 10-fold, filter-sterilized, and stored at 4°C until used. Based on the sizes of the toxins (308 kD for toxin A and 269 kD for toxin B), no significant loss of toxin protein during the concentration step was assumed, and a concentration of 500 μg/ml of each inactivated toxin in the 10x solution was estimated. The toxoid material was devoid of any detectable cytotoxic activity against IRM-90 cells (ATCC accession number CCL 186).

Preparation of a Whole Cell Vaccine

C. difficile VPI strain 10463 (ATCC accession number 43255) was grown in proteose peptone-yeast extract media (PPY; Holbrook, *et al.* J. Appl. Bacteriol. 42:259-273, 1977) at 37°C for 36 hours under anaerobic conditions to minimize spore formation. The cultures were centrifuged and the pelleted cells were washed 3 times with PBS. After the final wash, the pelleted cells were resuspended in PBS containing 1% (vol:vol) formaldehyde and incubated at 4°C for 24 hours. Excess formaldehyde was removed by 3 washes with PBS, and the

formalinized *C. difficile* cell suspension was stored at 4°C. Inoculation of the equivalent of 10^9 *C. difficile* colony-forming units (CFU) (a cell suspension with an O.D. of 1.0 at 550 nm) into PPY media yielded no growth after 36 hours of culture at 37°C under anaerobic conditions.

Animals

Female Syrian hamsters (*Mesocricetus auratus*, Charles River, Kingston, NY), 6-8 weeks old at the time of immunization, were used in all of the experiments. The animals were caged in groups of 5 during the immunization period, and then caged individually during *C. difficile* challenge.

Immunization Regimens

Seven different immunization regimens were analyzed (Table 1). For intranasal (i.n.) immunization, 5 μg of each toxoid (inactivated Toxin A and inactivated Toxin B), in 10 μl of the 10x toxoid, were mixed with a 5 μl solution containing 5 μg of cholera toxin (Calbiochem, La Jolla, CA). The 15 μl antigen-adjuvant mixture was administered into the external nares of the hamsters with a micropipettor, with half of the dose administered to each nostril. For intragastric (i.g.) immunization, 100 μg of each toxoid were mixed with 10 μg of cholera toxin, adjusted to a volume of 1 ml with PBS, and administered by gavage. For intraperitoneal (i.p.) and subcutaneous (s.c.) immunizations, 5 μg of each toxoid were mixed with 0.3 ml of RIBI adjuvant (RIBI, ImmunoChem, Hamilton, MT). For rectal (r.) immunization, 50 μg of each toxoid, in 100 μl of toxoid, were mixed with a 1 μl solution containing 10 μg of cholera toxin. For rectal administration of whole cells (w.c.r.), 5 x 10^8 cells were mixed with 50 μg of each toxoid, in 100 μl of toxoid, plus a 1 μl solution containing 10 μg cholera toxin. For both r. and w.c.r. groups, the sample was

applied with a disposable 20 x 1½ feeding needle inserted 3 cm into the rectum. The i.n., i.g., i.p., and s.c. immunizations were performed in animals lightly anaesthetized with isofluorane. The r. and w.c.r. immunizations were done in pentobarbital anaesthetized animals. A control intranasal group (c.i.n.) received 5 μg of cholera toxin intranasally. A control subcutaneous group (c.s.c.) received 0.3 ml of RIBI adjuvant subcutaneously. Groups of 5 animals were used for all immunization regimens. All groups received a total of 4 doses of the vaccine (or adjuvant control) on days 0, 7, 14, and 28 of the experiment.

Table 1. Schemes of immunization of hamsters with formalin-inactivated *C. difficile* cultures (inip = intranasal + intraperitoneal; in = intranasal; ig = intragastric; r = rectal; wcr = whole-cell rectal; ip = intraperitoneal; sc = subcutaneous; cin = control intranasal; csc = control subcutaneous).

Antigen	Route of immunization (group)	Dose per immunization	Adjuvant, dose per immunization
Culture filtrate toxoid	intranasal, 1st 3 immunizations; intraperitoneal + intranasal, 4th immunization (inip)	5 μg toxin A, 5 μg toxin B	5 μg cholera toxin for in; 0.3 ml RIBI for ip
Culture filtrate toxoid	intranasal (in)	5 μg toxin A, 5 μg toxin B	5 μg cholera toxin
Culture filtrate toxoid	intragastric (ig)	100 μg toxin A, 100 μg toxin B	10 μg cholera toxin
Culture filtrate toxoid	rectal (r)	50 μg toxin A, 50 μg toxin B	10 μg cholera toxin
Culture filtrate toxoid and formalin killed *C. difficile* cells	rectal (wcr)	50 μg toxin A, 50 μg toxin B, 5 x 10^8 cells	10 μg cholera toxin
Culture filtrate toxoid	intraperitoneal (ip)	5 μg toxin A, 5 μg toxin B	0.3 ml RIBI
Culture filtrate toxoid	subcutaneous (sc)	5 μg toxin A, 5 μg toxin B	0.3 ml RIBI
phosphate buffer	intranasal (cin)	15 μl	5 μg cholera toxin
phosphate buffer	subcutaneous (csc)	0.3 ml	0.3 ml RIBI

To evaluate the immune responses, samples (200-400 μl) of blood were obtained on days 0, 2, 4, 7, and 36 from the retro-orbital sinus of the hamsters under isofluorane anesthesia. The blood was left to clot overnight at 4°C, and the serum was obtained by centrifugation. Only serum antibodies were evaluated; secretory IgA was not measured because of a lack of a suitable anti-hamster IgA reagent. After *C. difficile* challenge, a sample of feces was obtained every other day from the surviving animals and mixed with 2 volumes of PPY media for evaluation of the degree of colonization and presence of toxins (see below).

***C. difficile* Challenge**

All hamsters were challenged on day 38 (10 days after the 4^{th} immunization) with 0.5 mg of clindamycin administered orogastrically, followed 3 hours later by an orogastric inoculation of 10^5 CFU of viable *C. difficile* 10463 strain (ATCC accession number 43255), which were washed with PPY media, in order to eliminate free toxins. After challenge, the hamsters were observed daily for diarrhea and illness. The severity of the diarrhea was scored as: 0, no diarrhea; 1+, loose feces, but no wet tail; 2+, peri-anal and tail region wet; and 3+, tail, paws, and lower abdomen wet (animals with this appearance were usually hunched and inactive).

Evaluation of Tissue Damage

Severely ill hamsters were euthanized. Samples of cecum from the euthanized hamsters, and from the survivors from every immunization regimen, taken 8 days after clindamycin challenge, were fixed in 10% neutral buffered formalin. Formalin-fixed tissues were embedded in paraffin, sectioned at 5 μM, stained with hematoxylin and eosin, and examined by light microscopy. Histologic grading criteria were: 0, minimal infiltration of lymphocytes, plasma cells, and eosinophils; 1+, mild

infiltration of lymphocytes, plasma cells, neutrophils, and eosinophils, plus mild congestion of the mucosa, with or without hyperplasia of gut associated lymphoid tissue; 2+, moderate infiltration of mixed inflammatory cells, moderate congestion and edema of the lamina propria, with or without goblet cell hyperplasia, individual surface cell necrosis or vacuolization, and crypt dilatation; and 3+, severe inflammation, congestion, edema, and hemorrhage in the mucosa, surface cell necrosis, or degeneration with erosions or ulcers.

Evaluation of Infections

Feces obtained after clindamycin challenge were studied for the presence of *C. difficile*. Ten-fold dilutions in PPY media were inoculated onto selective media containing cycloserine (125 μg/ml) and cefoxitin (8 μg/ml), and colonies were counted after 48 hours of incubation under anaerobic conditions. The presence of Toxin A in feces was determined using a Toxin A kit (TechLab, Blacksburg, VA), as described by the manufacturer. After 15 minutes with substrate, the O.D. was read at 450 nm, and the concentration of toxin was estimated from a standard curve of Toxin A prepared in each plate. The estimations were carried out using Softmax software (Molecular Devices, Sunnyvale, CA). For quantification of Toxin B, fecal suspensions were centrifuged and filter-sterilized, and ten-fold dilutions of the samples were tested for cytopathic effects on IMR-90 fibroblast cell cultures, as is described below.

ELISA for Antibodies to Toxin A and Toxin B

Microtiter plates (Corning, New York, NY) were coated with 100 ng/well of purified Toxin A or Toxin B in carbonate-bicarbonate buffer, pH 9.3, and incubated overnight at 4°C. The plates were washed and blocked with 2.5% non-fat dry milk (NFDM) in phosphate buffered saline solution (PBS), pH 7.4. Serum samples were added

at two-fold dilutions ranging from 1:500 to 1:64,000, and the plates were incubated for 1 hour at 37°C. Anti-hamster IgG (1:1000, Southern Biotech, Birmingham, AL) conjugated with alkaline phosphatase, was added, incubated for 1 hour at 37°C, and washed prior to addition of a p-nitrophenyl phosphate substrate. A positive control was included in each plate; wells were coated with Toxin A or Toxin B in two-fold dilutions ranging from 100 to 0.8 ng/ml, and reacted with specific goat anti-toxin (TechLab), followed by an anti-goat IgG alkaline phosphatase conjugate. Negative controls were wells coated with purified toxin and reacted with an anti-hamster IgG alkaline phosphatase conjugate. The O.D. was read at 405 nm, and the titer was defined as the reciprocal of the highest dilution of sample giving an O.D. $\geq$ 0.3.

ELISA for Antibodies to Whole Cell Antigens

Plates were coated with 100 μl of a formalin-killed *C. difficile* suspension adjusted to an O.D. of 0.2 at 550 nm, and then incubated overnight in an orbital shaker at 150 rpm. The cells were fixed to the plates by incubation at 70°C for 2 hours. After washing, serum samples were added at two-fold dilutions ranging from 1:100 to 1:12,800 and incubated for 1 hour at 37°C. Anti-hamster IgG and substrate were added as is described above. A positive control was included in each plate using mouse *C. difficile* whole cell antiserum at 1:500 to 1:64,000. (The antiserum was produced against VPI strain 10463, using standard methods). The wells coated with whole cells were reacted directly with the anti-hamster IgG alkaline phosphatase conjugate as negative controls.

Inhibition of Cytotoxicity

IMR-90 fibroblast cells were grown to confluence in 96-well plates in D-MEM media (Gibco, Grand Island, NY) containing 10% fetal calf serum. The minimal dose of

Toxin A or Toxin B needed to cause 100% rounding of the cells was defined as 1 cytotoxic unit (CTU_{100}). For Toxin A, 6.3 ng/ml, and for Toxin B, 125 pg/ml, were defined as 1 CTU_{100}. Two-fold dilutions of the hamster serum samples, ranging from 1:100 to 12,800, were mixed with $4CTU_{100}$ of either toxin, incubated for 1 hour at 37°C, and the mixture was then added to the cells. Goat anti-Toxin A and goat anti-Toxin B served as positive controls. Cells were observed after 24 hours, and the proportion of round cells was determined. The titers of the samples were defined as the reciprocal of the highest dilution of sera inhibiting ≥50% cell rounding.

Agglutination

Twenty-five μl samples of hamster serum were diluted ranging from 1:25 to 1:3,200. The dilutions were prepared in 96-well U-bottom microplates (Falcon, Oxnard, CA). The formalin-killed *C. difficile* suspension was adjusted to an O.D. of 1.0 at 550 nm, and 25 μl of the suspension were added to the serum dilutions. Mouse anti-*C. difficile* whole cell anti-serum served as a positive control, and PBS was used as a negative control. The plates were incubated overnight at 4°C, and the agglutination was then scored. Endpoint titers were defined as the reciprocal of the highest dilution of serum causing agglutination.

Western Blot Analysis

C. difficile VPI strain 10463 (ATCC accession number 43255), and the strains isolated from hamsters after clindamycin challenge were grown in 5 ml of PPY media at 37°C under anaerobic conditions for 36 hours. The cultures were centrifuged and the pellets were washed three times with PBS. The pellets were resuspended in 250 μl of 3% SDS in PBS, and the lysates were fractionated by electrophoresis in a 12% preparative SDS-polyacrylamide gel (Bio-Rad, Hercules, CA) at 200 volts

for 1 hour. Proteins were transferred from the gel to nitrocellulose at 150 volts for 1.2 hours in a Bethesda Research Laboratories Mini-V 8-10 chamber (Life Technologies, Grand Island, NY). The membranes were blocked with 5% non-fat dry milk in PBS for 1 hour, washed, and mounted in a multiscreen apparatus (BioRad, Hercules, CA). A 1:200 dilution of each hamster serum sample was then added and incubated for 1 hour, and the reaction was developed with NBT/BCIP (Gibco, Gaithersburg, MD). Mouse anti-*C. difficile* 10463 whole cell serum served as a positive control. To type *C. difficile* strains isolated from feces, SDS-lysates from isolates were fractionated by electrophoresis, transferred to nitrocellulose, and reacted with whole cell mouse antiserum, as is described above.

Statistical Analysis

The immune responses to the different *C. difficile* antigens was studied for possible significant correlation with the outcome of the hamsters after clindamycin challenge using the Kruskal-Wallis test (Quick-STATISTICA software, StatSoft, Tulsa, OK).

RESULTS

Outcome After Clindamycin Challenge

Hamsters were challenged with clindamycin and *C. difficile* 10 days after the last immunization. All sham-immunized, control intranasal (c.i.n.), and control subcutaneous (c.s.c.) animals died within 48 hours of challenge, most with severe (3+) diarrhea. Acute, diffuse necrohemorrhagic typhlitis (grade 3+) was found on pathologic examination. Crypt epithelium was hyperplastic and dilated crypts were filled with neutrophils. Lymphocytes, plasma cells, and neutrophils infiltrated the lamina propria. Vaccinated hamsters that succumbed to challenge also died within the first 48

hours, and most had grade 3+ diarrhea and grade 3+ typhlitis on histopathologic examination. Animals that survived challenge either had no diarrhea or had diarrhea ranging in severity from 1+ to 3+. The severity of diarrhea correlated with the severity of typhlitis on pathologic examination. Animals with 3+ diarrhea had subacute, diffuse mucopurulent typhlitis grades 2-2.5+. Neutrophils, lymphocytes, and plasma cells infiltrated the lamina propria, and multifocal crypt abscesses also were noted. Those animals with 2+ diarrhea had subacute to chronic, moderate typhlitis grade 1.5-2+. Animals with mild diarrhea (1+) had a mild lymphocytic typhlitis, grades 1.0-1.5+. Mild lymphocytic typhlitis grade 1+ was also evident in hamsters without diarrhea.

The outcome of the clindamycin challenge in all vaccine groups is shown in Fig. 1. Mucosally immunized animals that received toxoid vaccine by the intragastric (i.g.) or rectal (r.) routes, or whole cell-toxoid vaccine by the rectal route (w.c.r.), were minimally protected against death, and all had diarrhea. The r. and w.c.r. immunization regimens protected only 20% of hamsters, while the i.g. regimen protected 40% of hamsters against death. Parenterally immunized (i.p. and s.c.) animals were fully protected against diarrhea. A similar outcome was observed in animals intranasally immunized (i.n.). When i.n. immunized animals received a booster dose of toxoid intraperitoneally (group i.n.i.p.), 100% protection was achieved against both death and diarrhea.

Presence of *C. difficile* and Toxins in Feces

Fecal samples obtained after clindamycin challenge were analyzed for the presence of *C. difficile*, Toxin A, and Toxin B. A similar pattern was observed in all surviving animals. Two days after challenge, *C. difficile* reached approximately 10^9 CFU/ml in feces;

thereafter, colonization decreased slightly and remained at about 10^8 CFU/ml for at least 9 days, regardless of the presence of diarrhea. In contrast, the levels of Toxin A and Toxin B steadily decreased, and by day 9, almost no toxins were found in feces, in spite of continuous colonization. All isolated *C. difficile* strains were typed by Western blot analysis using whole cell antiserum against the VPI 10463 strain (ATCC accession number 43255). Strains isolated from different animals and from different immunization groups were analyzed, and were all found to be similar to one another, suggesting that only one strain was colonizing all hamsters. However, this strain was different from VPI 10463, which is the strain used for challenge.

Immune Responses

Serum antibodies against *C. difficile* antigens were measured in hamsters from all experimental groups. Immune responses to Toxin A after the priming immunization were studied by ELISA in some of the groups. No specific IgG was detected in animals vaccinated by the i.n.i.p., r., and w.c.r. routes on days 2, 4, and 7 after the initial vaccine dose. In the parenterally immunized animals (i.p. and s.c.), no response was evident after days 2 and 4, but at day 7, a slight rise in antibody titer was observed. In contrast, the antibody responses measured after the last vaccine dose (day 36) demonstrated sero-conversion in all groups. The absence of early (anamnestic) antibody responses to the first vaccine dose shows that the animals were immunologically naive and had not previously been primed with Toxin A.

Three approaches were used to study the systemic antibody responses to *C. difficile* antigens: 1) recognition of immobilized antigens by ELISA; 2) inhibition of cytotoxicity in a cell culture assay; and 3) agglutination of bacteria. Toxin A, Toxin B, and

whole cells were used as antigens. Antibody responses to Toxin A, Toxin B, and whole cell antigen were present in all groups, as determined by ELISA (Fig. 2). Hamsters immunized by the i.n.i.p., i.n., i.g., and i.p. regimens had higher responses against Toxin A and Toxin B, than those immunized by the rectal (r. and w.c.r.) and s.c. routes. Antibody levels against whole cell antigens showed a pattern similar to that observed with toxins.

Antibodies to whole cell antigens were further characterized by Western blot analysis with whole cell lysates from the *C. difficile* strain isolated from the hamsters after clindamycin challenge. Animals in all immunization regimens developed antibodies to a 70 kD protein and to proteins with sizes of ≥200 kD (which are likely to be the toxins); animals immunized by the i.n.i.p. route had the strongest immune responses. A variety of other proteins were recognized by sera from animals immunized by the i.n.i.p., i.p., and s.c. routes, but these proteins were not apparent, or were less prominent, in sera from animals immunized mucosally (r. and w.c.r. groups). In other assays, hamster serum was immunoblotted in parallel against purified toxins and whole cell lysates. These assays demonstrated that the lower molecular weight proteins which reacted with the hamster sera were not toxin fragments.

Serum antibodies with biological functions showed a different pattern from that obtained by ELISA (Fig. 3). Antibodies inhibiting cytotoxicity by Toxin A were elicited in all animals. Hamsters immunized by the i.n.i.p. and i.p. routes developed the highest anti-toxin A activity (mean +SE 22,000±4,900 and 18,000±2,000, respectively), whereas mucosally immunized animals (i.n., i.g., r. and w.c.r.) had lower activities (580±280, 280±146, 1720±560, and 2760±290, respectively). High anti-Toxin B responses were obtained in all groups,

except in rectally immunized animals. Agglutinating antibodies were elicited only in animals that received toxoid vaccine parenterally (i.p. and s.c.), or by a combined mucosal-parenteral route (i.n.i.p.).

Correlation of the Immune Response and Protection

The i.n.i.p. immunized animals were fully protected against death and diarrhea and had the highest serum immune responses when both ELISA and biological activity were considered (Figs. 2 and 3). Complete protection against death was provided by all immunization schemes that included parenteral injection of the vaccine or intranasal immunization alone. In contrast, rectally immunized animals had the lowest protection ratios and serum antibody responses, particularly neutralizing antibody against Toxin B (Fig. 3). Immunological correlations were not consistent, however, as illustrated by the similar antibody responses in i.n. and i.g. groups (Figs. 2 and 3), despite the greater protection afforded by the i.n. vaccine (Fig. 1).

To define the immunologic correlations, animals from all groups were analyzed together, and the outcome of the challenges was compared with the immune responses (Table 2). Mean antibody levels in all tests, except the whole cell ELISA, were significantly higher in survivor animals than in animals with a lethal outcome. Hamsters with severe diarrhea (3+) had significantly lower serum immune responses by all assays, as compared to those animals without diarrhea (Table 3). Antibody responses in hamsters with mild-moderate diarrhea (1+ and 2+) did not differ significantly from those without diarrhea, except for the agglutinating antibody responses in those with 1+ diarrhea ($p<.05$).

Table 2. Correlation between immune response against *C. difficile* antigens and protection against death

Immunoassay	Total studied (n=34)	Antibody titer according to: death (n=11)	Antibody titer according to: survival (n=23)	
		Mean ± SE	Mean ± SE	p value[a]
Toxin A ELISA	5210±960	2750±1360	6390±1180	.0290
Toxin B ELISA	8610±1760	3800±2840	10910±2050	.0063
Whole-cell ELISA	6560±1890	8363±5610	5704±980	.1382
Anti-cytotoxin A	7370±1620	1470±390	10195±2170	.0001
Anti-cytotoxin B	8690±1900	1790±1240	9330±2610	.0197
C. difficile agglutination	130±25	25±10	180±31	.0032

[a]Kruskal-Wallis test

Table 3. Correlation between immune response against *C. difficile* antigens and severity of diarrhea

	no diarrhea (n=10)	diarrhea, 3+ (n=9)	
Immunoassay	Mean ± SE	Mean ± SE	p value[a]
toxin A ELISA	9400±1970	1333±160	.0003
toxin B ELISA	17,800±3430	870±230	.0001
whole cell ELISA	9000±1660	1820±310	.0001
anti-cytotoxin A	15840±3690	3220±610	.0016
anti-cytotoxin B	11540±4770	670±440	.005
C. difficile agglutination	265±49	55±28	.0098

[a]p values when compared with the no diarrhea group, Kruskal-Wallis test.

Long Term Protection

Four surviving animals from the i.n.i.p. group and four from the s.c. group were held for a period of 140 days after clindamycin challenge. On day 140, samples of blood and feces were taken, and the animals were re-challenged with clindamycin. Three of four animals (75%) in each group survived re-challenge. Two of four animals (50%) in the i.n.i.p. group and 0/4 (0%) in the s.c. group were protected against diarrhea. The immune responses before re-challenge were compared with the responses obtained before the first challenge. In the ELISA analysis, Toxin A and Toxin B antibodies were not reduced, although levels against whole cell antigens were markedly decreased (Fig. 4). When the biological activities were compared, marked decreases in anti-cytotoxin activity and in *C. difficile* agglutination were observed prior to re-challenge (Fig. 5).

Example II. Immunization of Mice with Vaccine Compositions Containing *C. difficile* Toxins

The following methods were used to analyze the efficacy of the immunization methods of the invention in the mouse model system.

ELISA

The ELISA methods used in the following experiments are described above. Briefly, toxin-specific immune responses were detected by coating 96-well plates with Toxin A or Toxin B, blocking the wells with skim milk in PBS-tween, addition of samples from the mice, and detection with a commercial anti-mouse alkaline phosphatase (AP) conjugated reagent. The plates were developed with Sigma 104 alkaline phosphatase (AP) substrate (Sigma Chemical Company, St. Louis, MO). Data from these assays are represented as the absorbance at 405 nm (see below).

Cytotoxicity

Both Toxin A and Toxin B mediate cytotoxicity against a variety of cell lines. The toxicity is manifested by the rounding of fibroblast cells (IMR-90). IMR-90 cells are sensitive to 10-100 pg of Toxin A and 0.1-1.0 pg of Toxin B. The dosage of toxins used in cytotoxicity inhibition experiments corresponds to 8x the amount required to cause rounding of 50% of a confluent monolayer of IMR-90 cells. Serum or secretions were diluted appropriately and mixed with either Toxin A or Toxin B for 1 hour at 37°C. The toxins were then added to confluent wells of a 96-well cell culture plate and incubated overnight. Plates were read using a phase contrast microscope. Data are presented as the highest dilution that protects 50% of the monolayer from rounding.

Vaccine Preparation

Toxoid and recombinant Toxin A (GST-ARU) were prepared as is described above.

Enterotoxicity

Toxin A enterotoxicity was assessed using ligated intestinal loops challenged with Toxin A. Antibodies inhibiting enterotoxicity were measured by challenging loops with Toxin A pre-incubated with sera or secretions containing antibodies. Rats were fasted prior to use, anesthetized, and sections of intestine were tied off. Each loop contains intact blood vessels and is free of feces. Toxin A (1-10 μg) was administered to the lumen of each loop, with and without pre-treatment with immune sera. After 4 hours, the loops were removed and the contents weighed. Mouse and hamster loops can be directly challenged in a similar fashion to determine the efficacy of immunization against enterotoxicity. In the mouse loop assay, the volume in PBS treated loops is

compared to Toxin A treated loops. The data is presented as the mg of contents per cm of ligated loop.

Systemic Challenge

Mice were challenged with 10x the LD_{50} of each toxin administered intraperitoneally. The data shown in the figure (see below) is the % animals surviving the challenge. Hamsters were challenged with 2 mg clindamycin and 1 x 10^7 vegetative *C. difficile* organisms.

RESULTS

Intranasal Immunization of Mice with Toxoid

Groups of 5 female Swiss Webster mice (Taconic Farms, Germantown, NY) were immunized weekly by the intranasal route with toxoid (15 μg of each toxin), with or without 5 μg CT as a mucosal adjuvant. The immunization scheme is summarized in Table 4. Serum, saliva, feces, and vaginal secretions were obtained after immunization. Specific IgA and IgG antibodies can be detected in serum, and specific IgA antibodies against both Toxin A and Toxin B can be detected in saliva, feces and on mucosal surfaces against both Toxin A and Toxin B after immunization (Figs. 6A and 6B). This response was apparent regardless of the administration of CT along with the toxoid. Serum from immunized animals also inhibited the cytotoxicity of both Toxin A and Toxin B (Figs. 7A-7C). Immune sera was used to passively protect rat loops from the enterotoxic effects of Toxin A (Fig. 8). Animals were challenged with 10 LD_{50} of Toxin A followed by 10 LD_{50} of Toxin B one week later. All immunized animals survived this challenge, while controls did not (Fig. 9). Finally, ligated intestinal loops from immunized animals were challenged with Toxin A, directly demonstrating the induction of antibodies, which were probably mucosal IgA antibodies, capable of inhibiting

fluid accumulation and, presumably, diarrhea (Fig. 10). These data demonstrate the *C. difficile* vaccine elicits a strong protective mucosal immune response when administered to a mucosal surface, and that does not require a mucosal adjuvant.

Table 4. Mucosal immune response after intranasal immunization of mice with *C. difficile* toxoid, +/- cholera toxin (CT); Immunizations were carried out on days 0, 7, 21, 35, 49, and 59. Challenge with toxin A/B took place on day 70. Samples (serum, feces, saliva, and vaginal secretions) were taken on day . (CT = cholera toxin; PBS = phosphate-buffered saline)

Antigen	Dose (μg)	Adjuvant	# Animals
Toxoid	15	5 μg CT	5
Toxoid	15	none	5
PBS	0	5 μg CT	5

Example III. Immunization of Mice with Vaccine Compositions Containing GST-ARU fusion proteins

The COOH-terminal region of *C. difficile* Toxin A contains a series of repeating amino acid units which are thought to be involved in binding of the toxin to carbohydrate residues on target cells (see, *e.g.*, Lyerly *et al.*, Current Microbiology 21:29-32, 1990; Frey *et al.*, Infection and Immunity 60:2488-2492, 1992; and references cited therein). A fusion protein consisting of the carboxyl-terminal region of *C. difficile* Toxin A fused to glutathione S-transferase (GST) was constructed, as follows. Using standard methods, a *Sau3A* fragment containing nucleotides which encode the 794 carboxyl-terminal amino acids of Toxin A was isolated (see, *e.g.*, Dove *et al.*, *supra*, for the sequence of the Toxin A gene). The sticky ends of the fragment were filled in, and the blunt-ended fragment was ligated into *SmaI*-digested pGEX3X. Clones containing a plasmid encoding GST-ARU were grown in *E. coli*, and the GST-ARU fusion protein was purified on a glutathione-agarose affinity

column, eluted from the column by free glutathione, and dialyzed to remove the glutathione, using standard methods.

Groups (n=5) of female Swiss Webster mice were immunized with Toxin A fusion protein (GST-ARU) by the intragastric (IG; 100 μg), intranasal (IN; 50 μg), or intraperitoneal (IP; 25 μg) routes, with or without CT (5 μg) as a mucosal adjuvant, in four weekly doses (Table 5). After the final dose, samples were obtained for immune analysis. All routes induced good serum immune responses against Toxin A. Intranasal (IN) administration resulted in mucosal IgA responses, even without CT. Immunization by the IG route was also effective, but seemed to be enhanced by the presence of a mucosal adjuvant. Immunization by the IP route induced good systemic and fecal responses, but not in salivary or vaginal samples (Figs. 11A-11B). Serum antibodies did not significantly inhibit the *in vitro* cytotoxicity of Toxin A, but were able to passively protect rat loops from enterotoxicity, suggesting that the carboxyl-terminal binding domain is only required for *in vivo* toxicity (Figs. 12A-12B and 13A-13B). Animals immunized by the IN and IP routes were protected from lethal challenge, but the IG immunized animals were not (Fig. 14). Survivors of the systemic challenge also demonstrated the presence of enterotoxin neutralizing antibodies in ligated intestinal loops (Fig. 15). IN immunization resulted in circulating antibodies that protected animals from the lethal effects of Toxin A, as well as the enterotoxic effects of Toxin A on the intestinal mucosa, even without CT. Some protection was observed by all routes tested, but the IN route of administration appeared to be more effective in eliciting a mucosal response.

Table 5. Mucosal immune response after immunization of mice with recombinant *C. difficile* toxin A COOH terminus (GST-ARU). Immunizations were carried out on days 0, 7, 14, and 21. Challenge with toxin A took place on day 35. Samples (serum, feces, saliva, and vaginal secretions) were taken on day 28. (CT = x μg cholera toxin; IG = intragastric; IN = intranasal; IP = intraperitoneal; PBS = phosphate-buffered saline)

Antigen	Dose (μg)	Adjuvant	Route	# Animals
GST-ARU	100	CT	IG	5
GST-ARU	100	none	IG	5
GST-ARU	50	CT	IN	5
GST-ARU	50	none	IN	5
GST-ARU	25	RIBI	IP	5
GST-ARU	25	none	IP	5
PBS	0	CT	IN	5

Other embodiments are within the following claims.

What is claimed is:

1. A composition comprising non-replicatable polypeptide antigen which is dissolved in a pharmaceutically acceptable diluent and which is capable of inducing a distal mucosal immune response to a gastrointestinal or genitourinary tract pathogen in a mammal, when administered intranasally.

2. The composition of claim 1, wherein said distal mucosal immune response is in the gastrointestinal tract.

3. The composition of claim 1, wherein said distal mucosal immune response is in the genitourinary tract.

4. The composition of claim 1, wherein said pathogen causes diarrhea.

5. The composition of claim 4, wherein said pathogen is from the genus *Clostridium*.

6. The composition of claim 5, wherein said pathogen is *Clostridium difficile*.

7. The composition of claim 1, wherein said antigen comprises a toxin of said pathogen, or an immunogenic fragment or derivative thereof.

8. The composition of claim 7, wherein said antigen comprises *Clostridium difficile* Toxin A, or an immunogenic fragment or derivative thereof.

9. The composition of claim 7, wherein said antigen comprises *Clostridium difficile* Toxin B, or an immunogenic fragment or derivative thereof.

10. The composition of claim 8, wherein said antigen comprises *Clostridium difficile* Toxin A and *Clostridium difficile* Toxin B.

11. The composition of claim 1, wherein said antigen comprises a toxoid.

12. The composition of claim 11, wherein said toxoid is a *Clostridium difficile* toxoid.

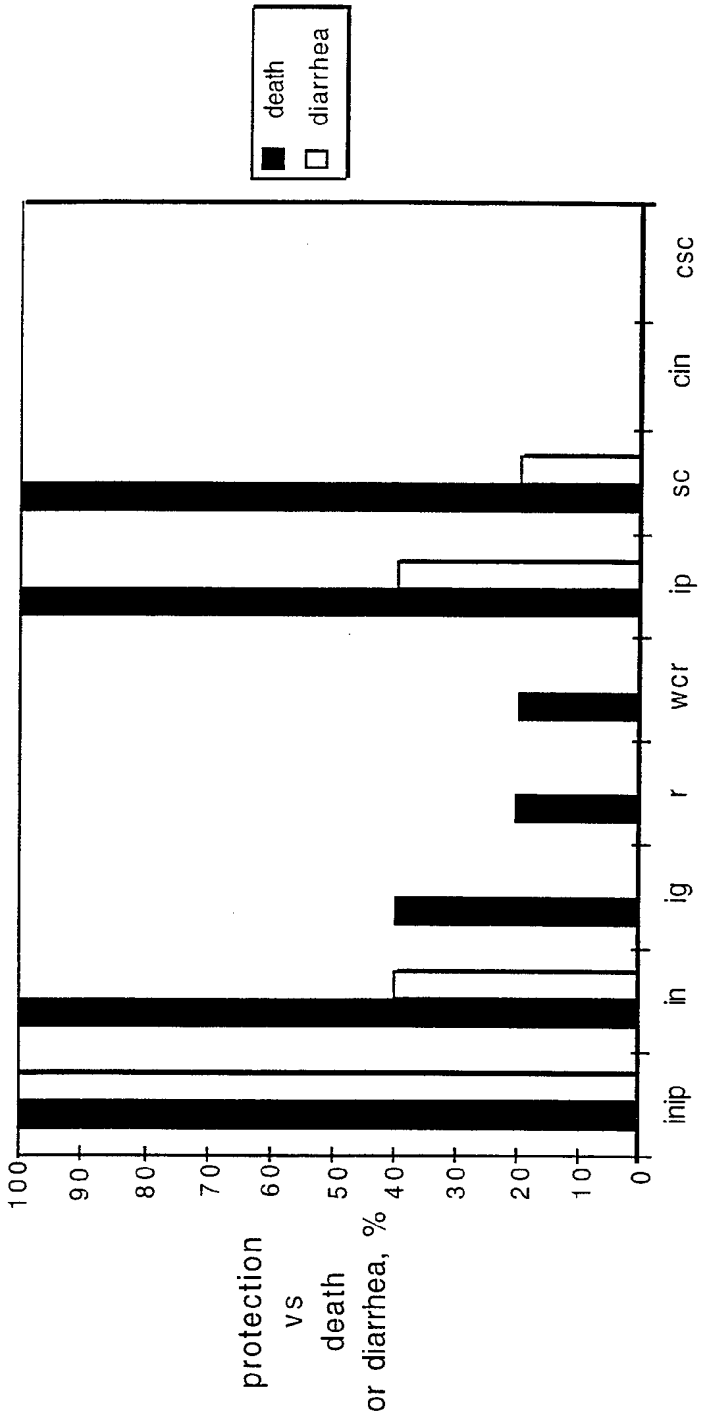
13. A method of inducing a distal mucosal immune response to a pathogen in a mammal, said method comprising the steps of:

a. administering an antigen capable of inducing said immune response to a mucosal surface of said mammal; and

b. parenterally administering said antigen to said mammal.

14. The method of claim 13, wherein said mammal is at risk of developing, but does not have, diarrhea caused by said pathogen.

15. The method of claim 14, wherein said antigen is a *C. difficile* toxoid.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diarrhea
protection vs death or diarrhea, %
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90
80
70
60
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inip
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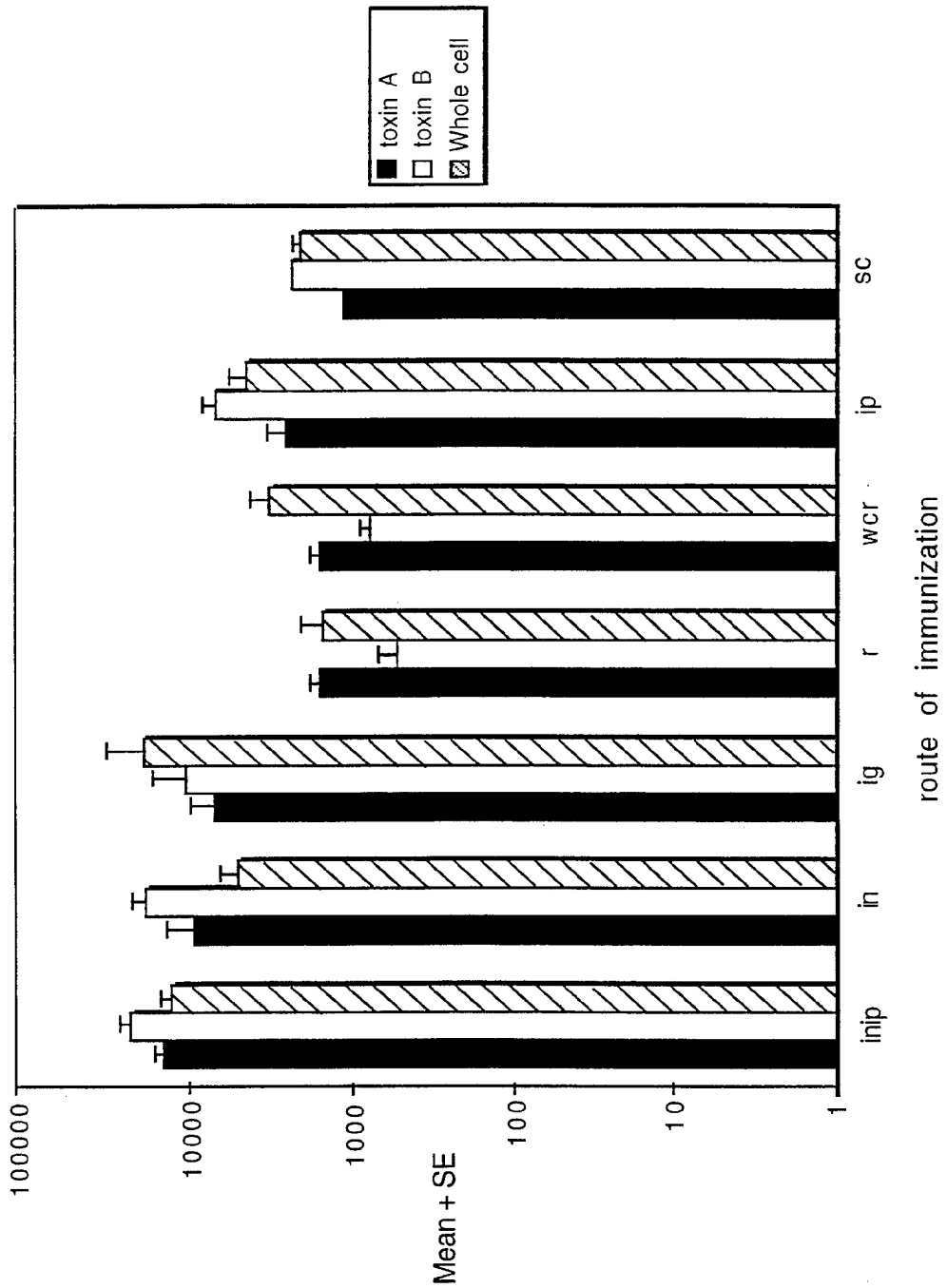
r
wcr
ip
sc
cin
csc
route of immunization

FIG. 1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toxin B
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10
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Mean + SE
inip
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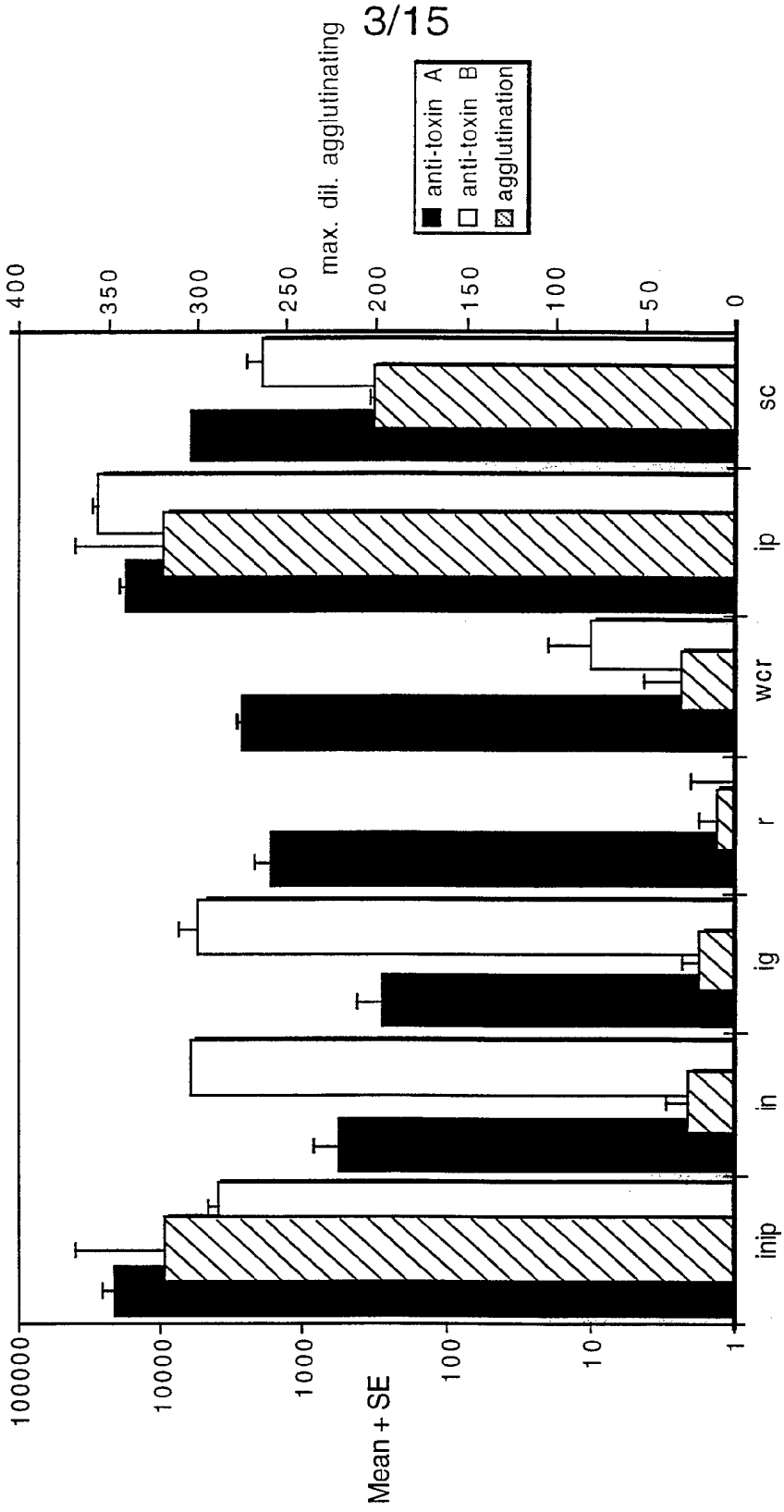
route of immunization

FIG. 2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anti-toxin A
anti-toxin B
agglutination
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350
300
250
200
150
100
50
0
Mean + SE
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10000
1000
100
10
1
inip
in
ig
r
wcr
ip
sc
route of immunization

FIG. 3

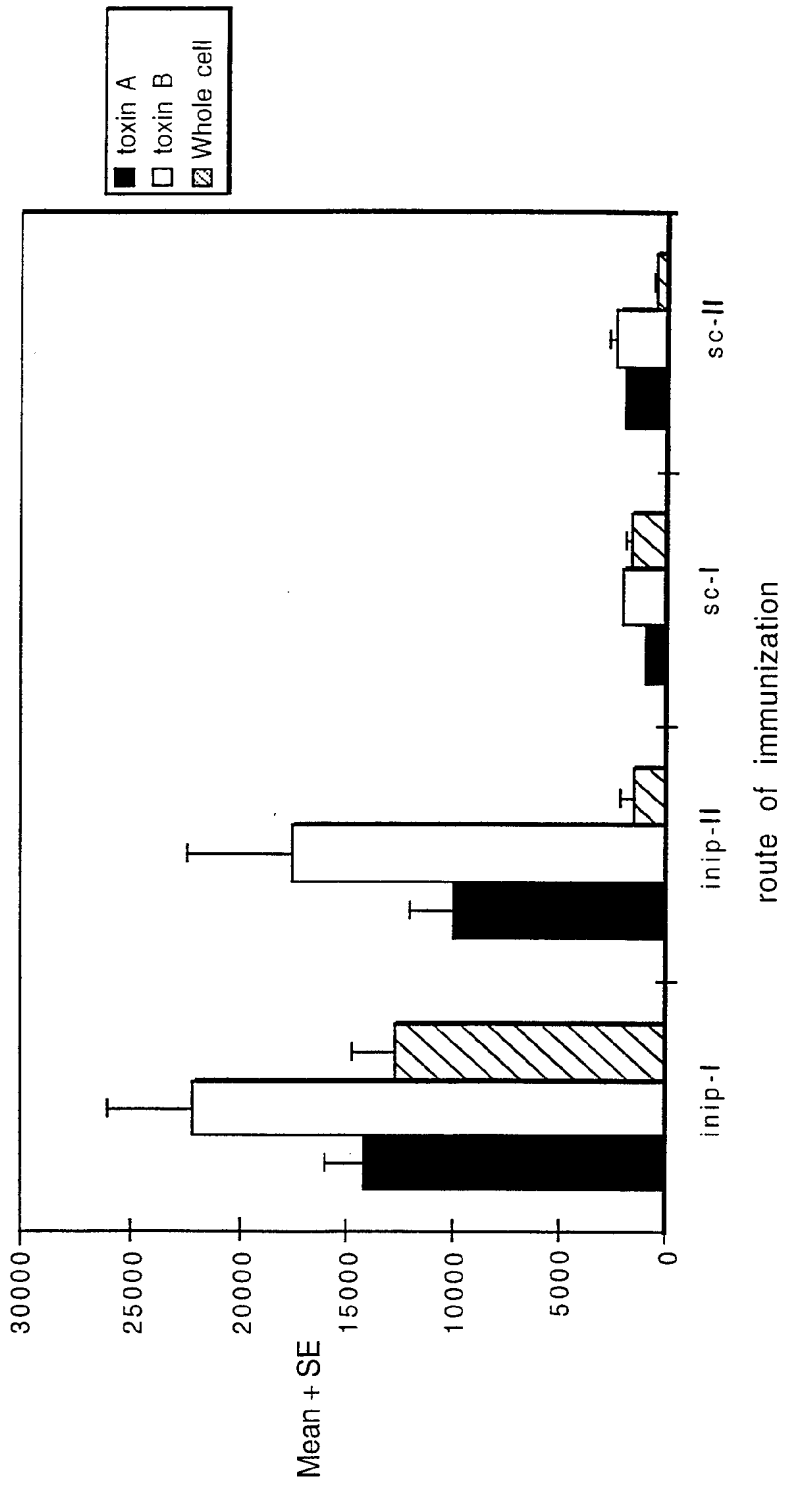

toxin A
toxin B
Whole cell
30000
25000
20000
15000
10000
5000
0
Mean + SE
inip-I
inip-II
sc-I
sc-II
route of immunization

FIG. 4

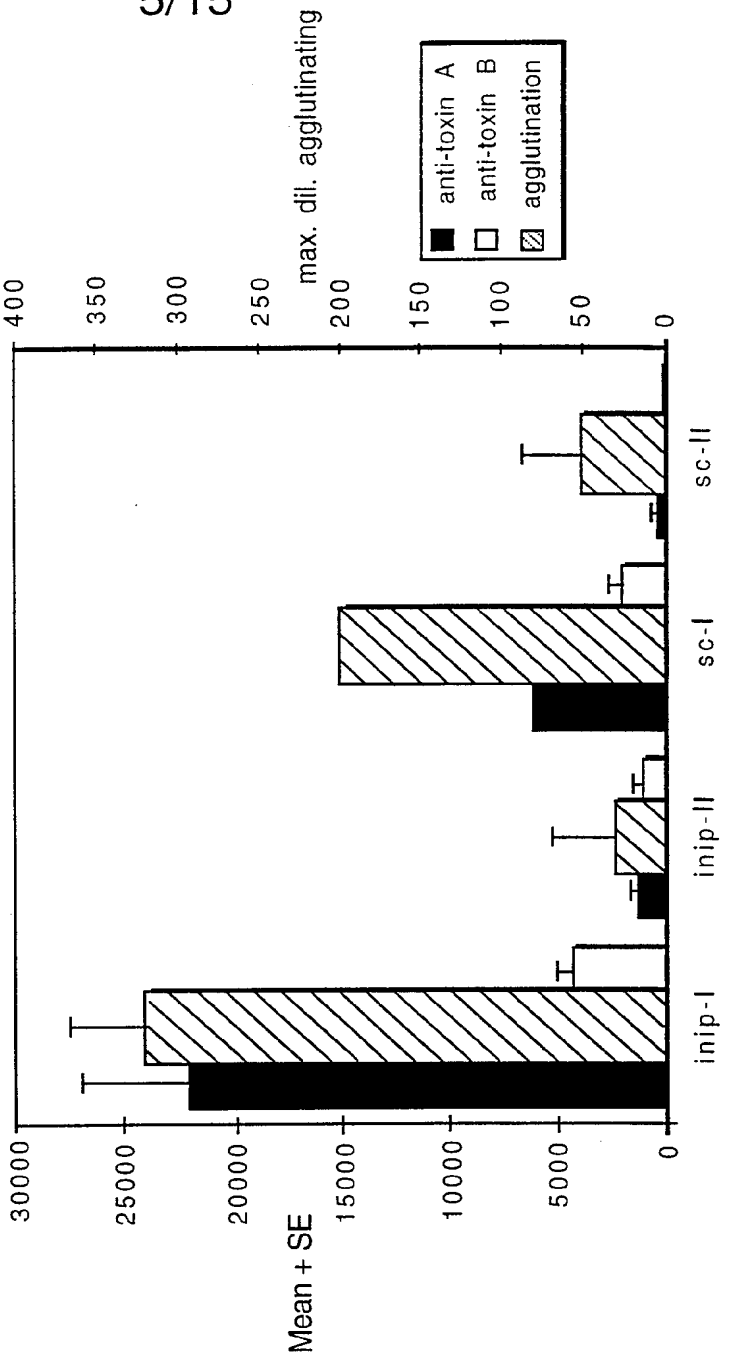

max. dil. agglutinating
400
350
300
250
200
150
100
50
0
anti-toxin A
anti-toxin B
agglutination
30000
25000
20000
15000
10000
5000
0
Mean + SE
inip-I
inip-II
sc-I
sc-II
route of immunization

FIG. 5

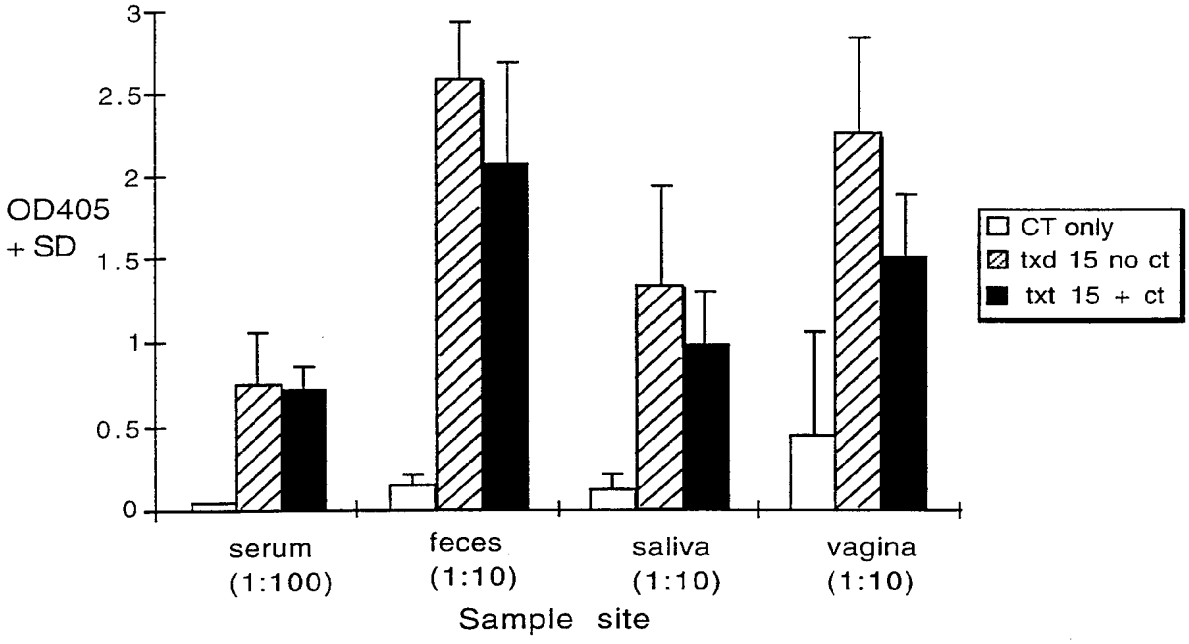

3
2.5
2
1.5
1
0.5
0
OD405
+ SD
CT only
txd 15 no ct
txt 15 + ct
serum
(1:100)
feces
(1:10)
saliva
(1:10)
vagina
(1:10)
Sample site

FIG. 6A

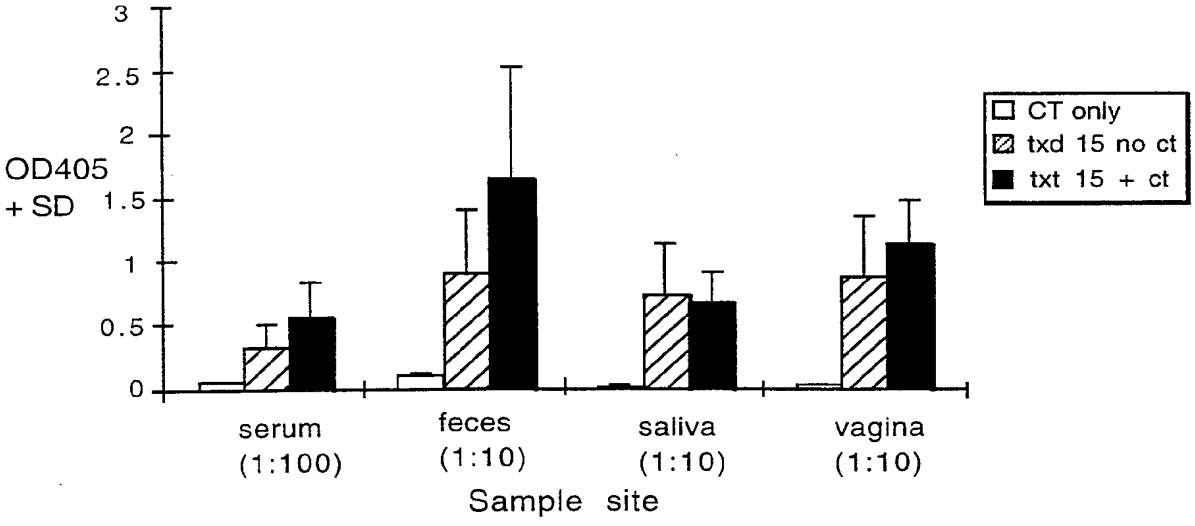

3
2.5
2
1.5
1
0.5
0
OD405
+ SD
CT only
txd 15 no ct
txt 15 + ct
serum
(1:100)
feces
(1:10)
saliva
(1:10)
vagina
(1:10)
Sample site

FIG. 6B

FIG. 7A

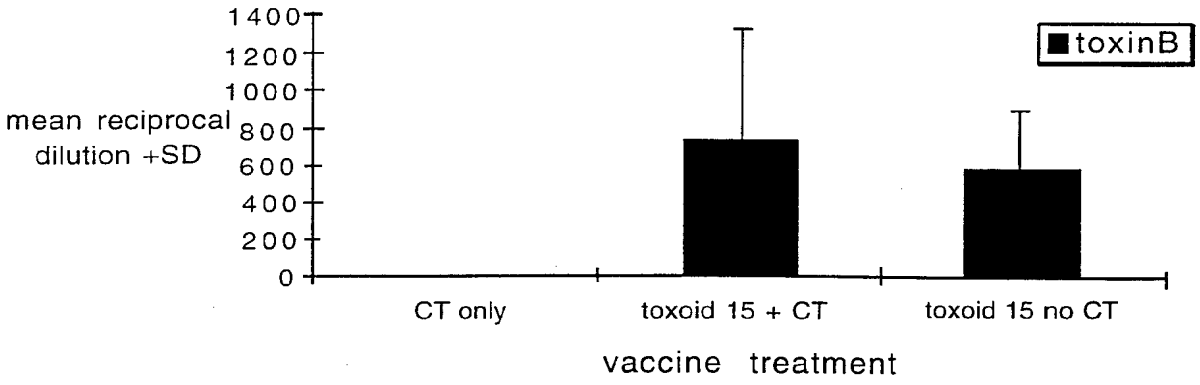


FIG. 7B

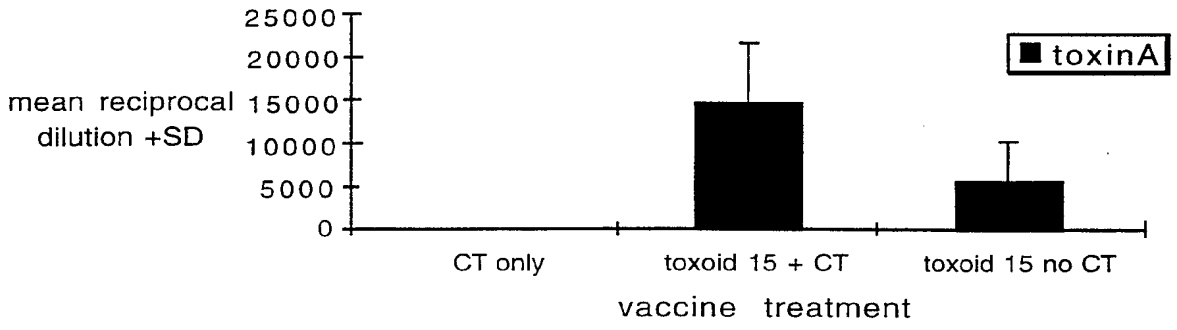
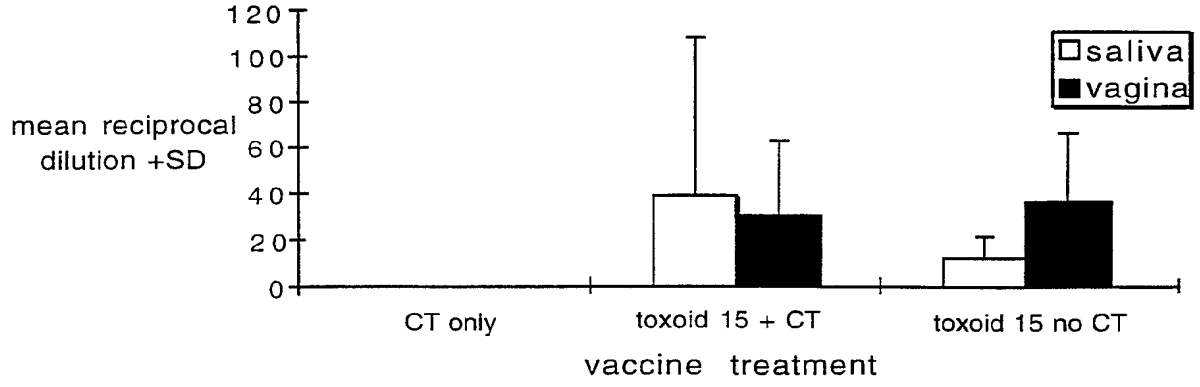


FIG. 7C



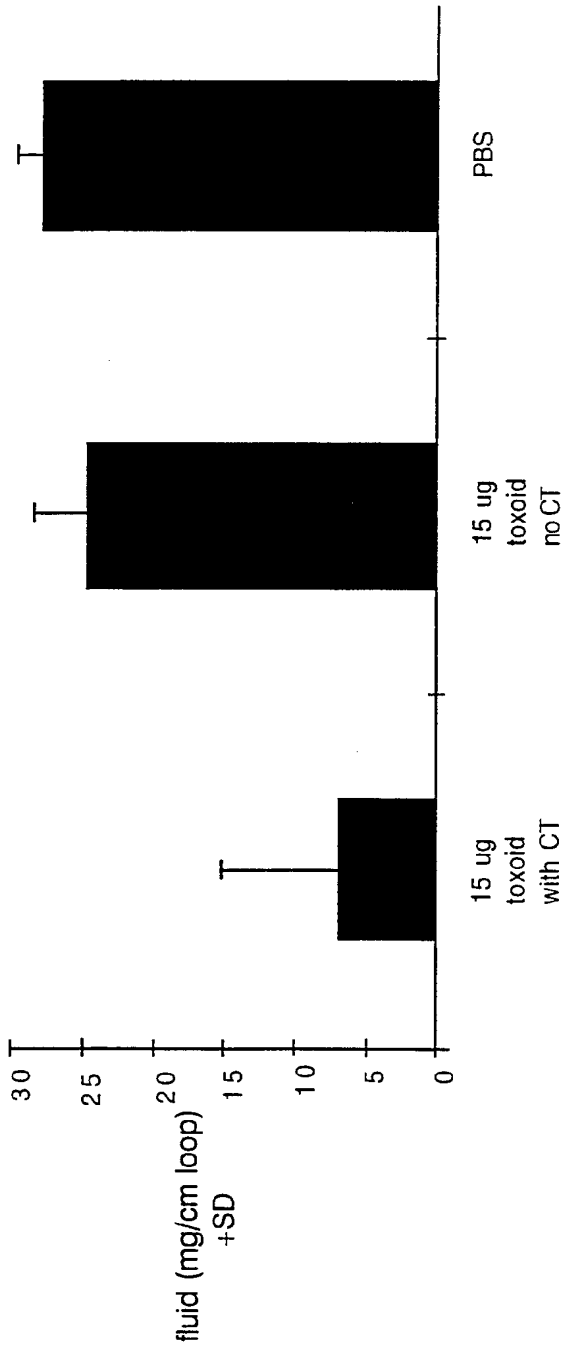

fluid (mg/cm loop) +SD
30
25
20
15
10
5
0
15 ug toxoid with CT
15 ug toxoid no CT
PBS
treatment group

FIG. 8

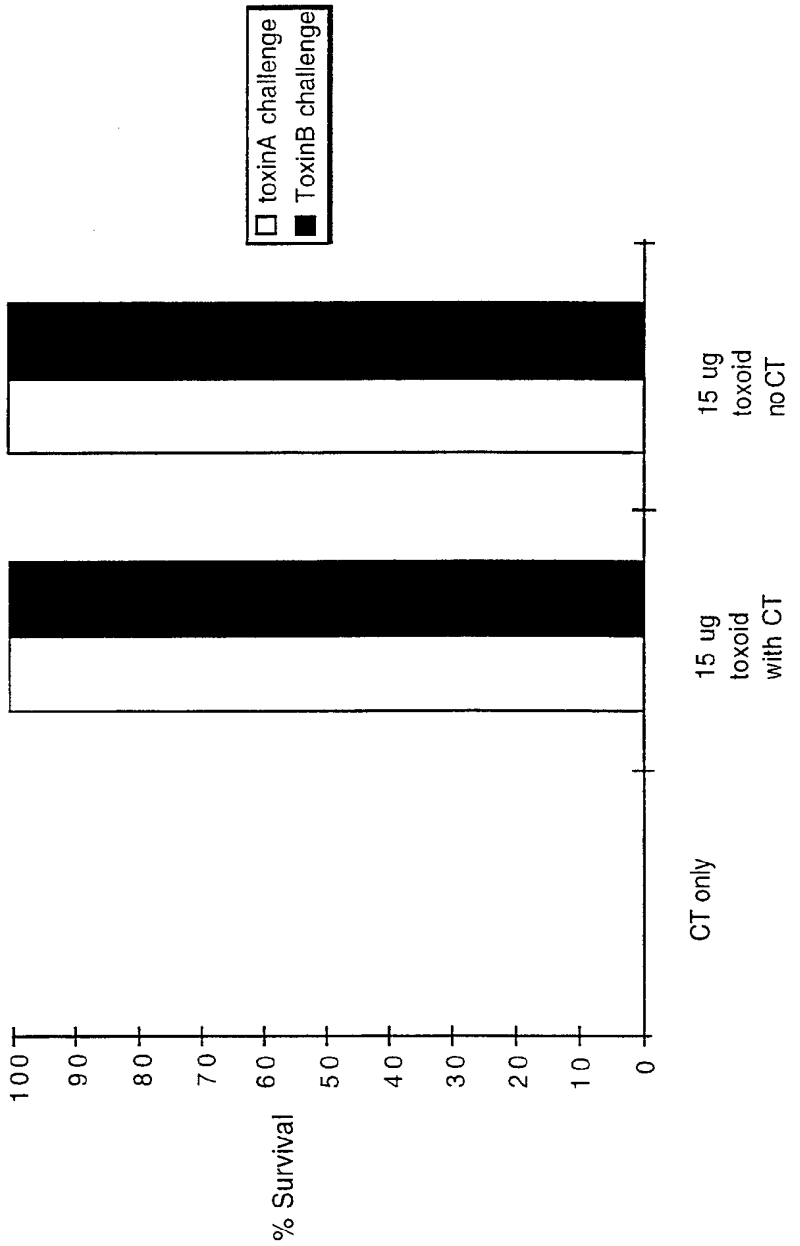

toxinA challenge
ToxinB challenge
% Survival
100
90
80
70
60
50
40
30
20
10
0
CT only
15 ug toxoid with CT
15 ug toxoid no CT
Vaccine treatment

FIG. 9

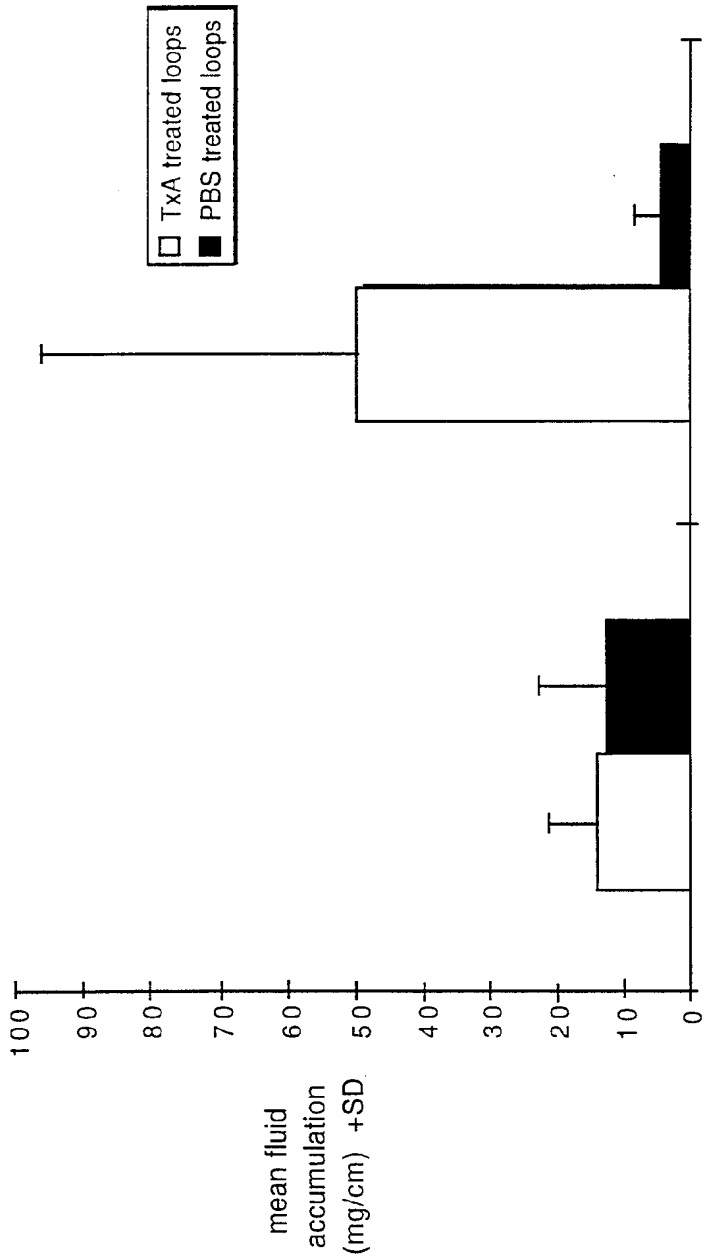

TxA treated loops
PBS treated loops
mean fluid accumulation (mg/cm) +SD
100
90
80
70
60
50
40
30
20
10
0
not treated
toxoid 15 ug IN + CT
treatment group

FIG. 10

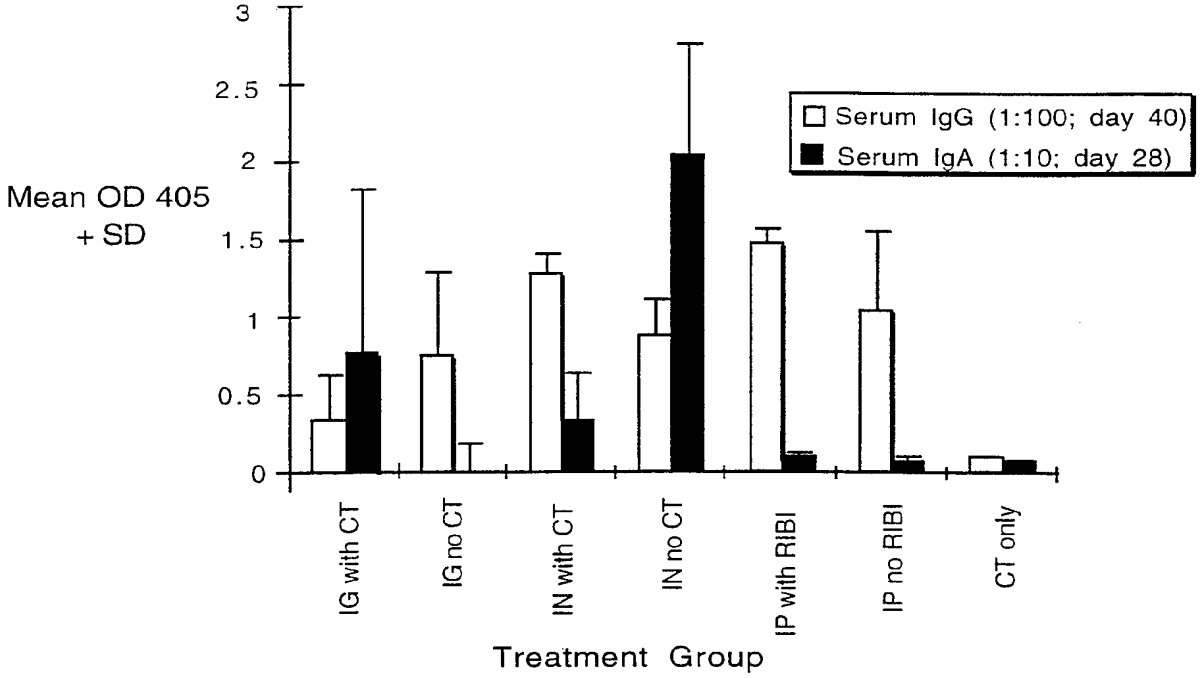


FIG. 11A

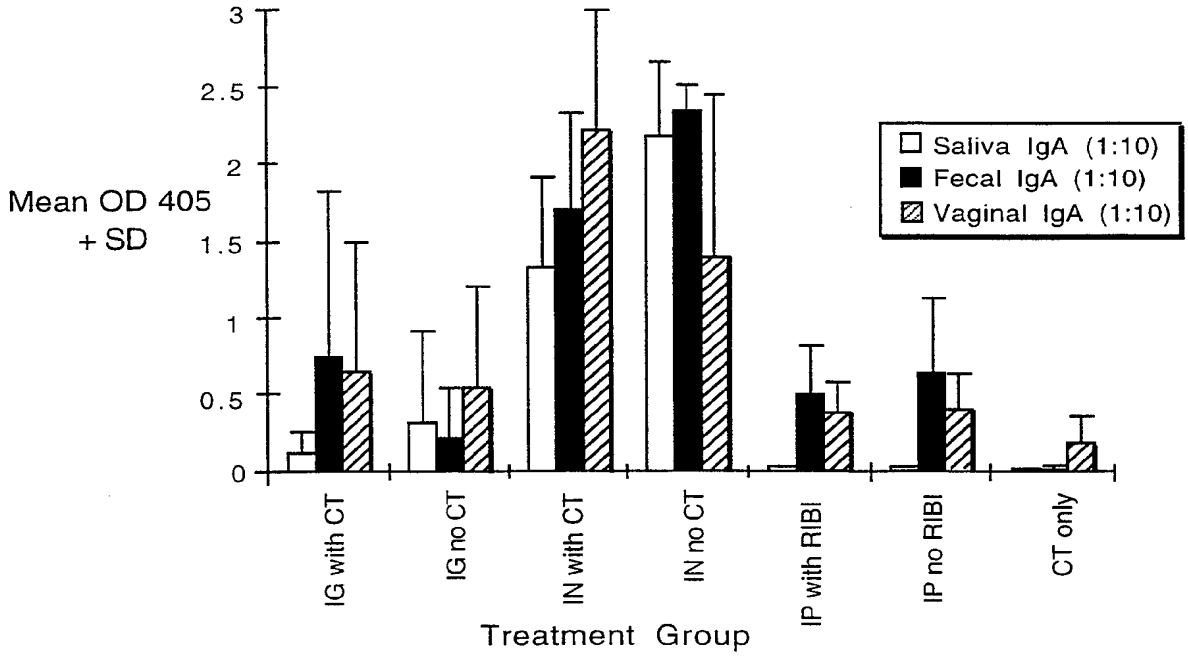


FIG. 11B

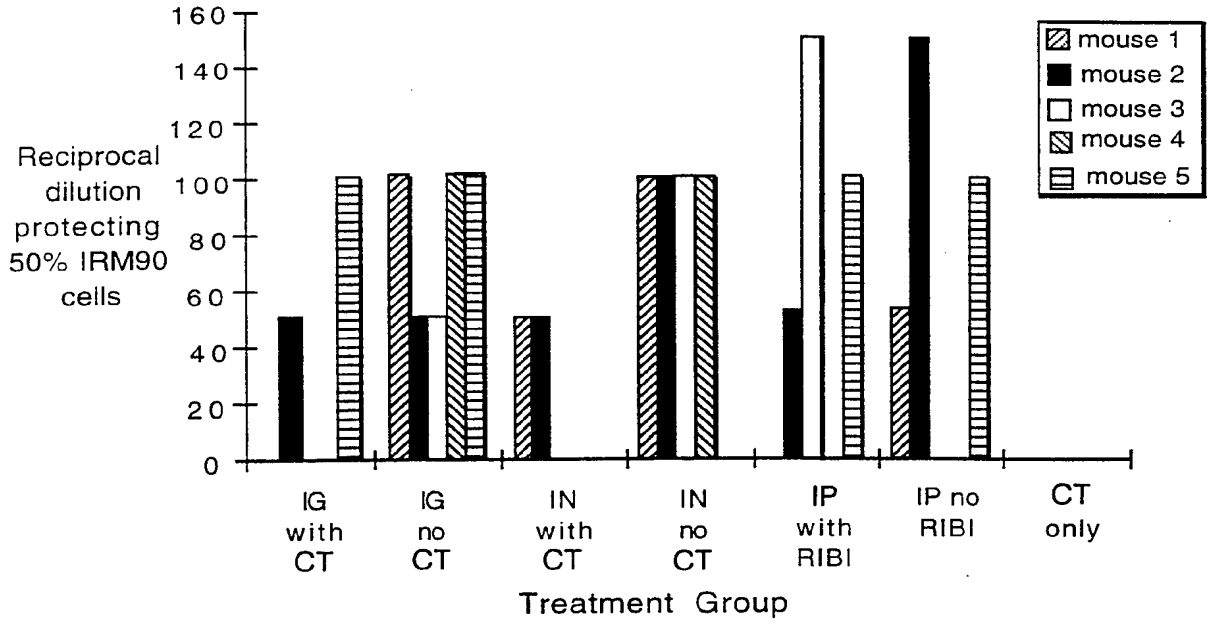


FIG. 12A

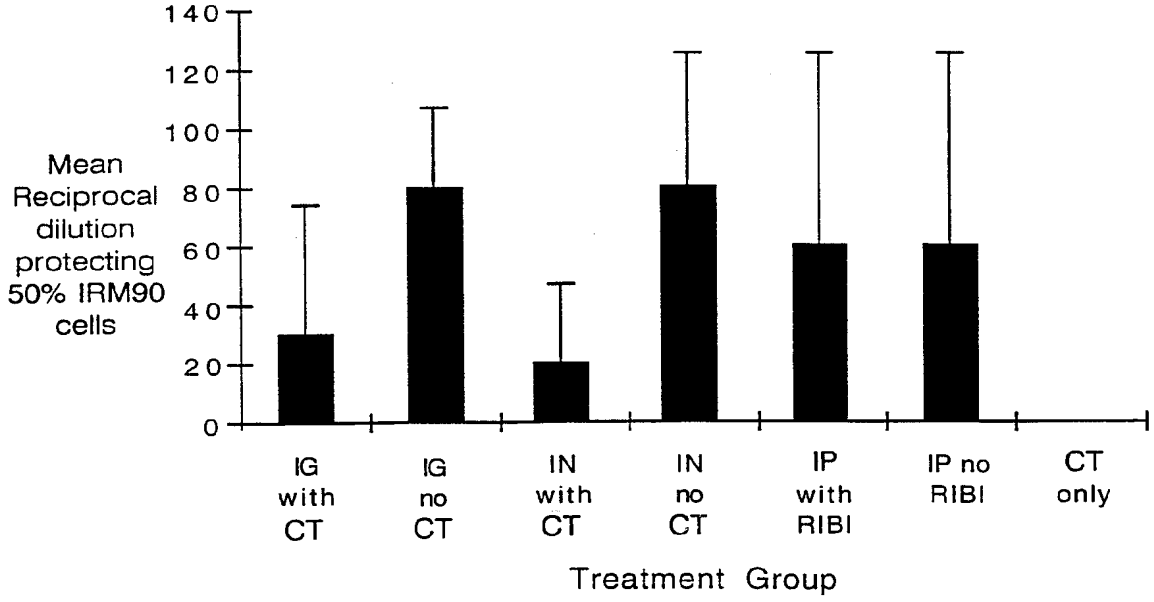


FIG. 12B

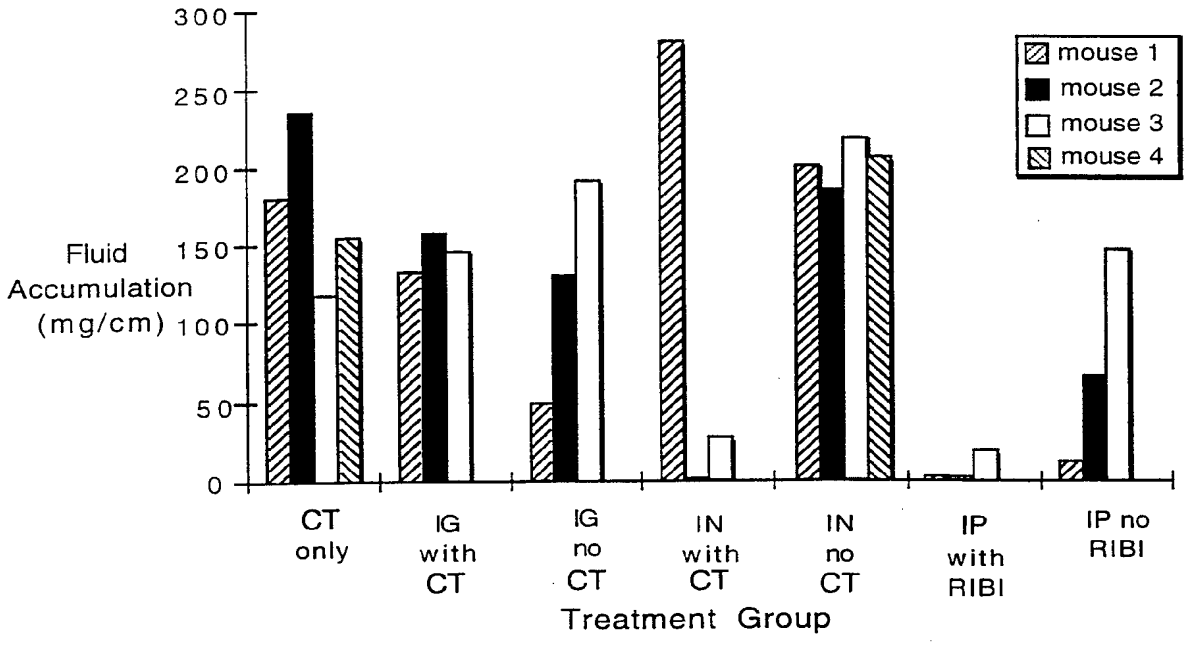


FIG. 13A

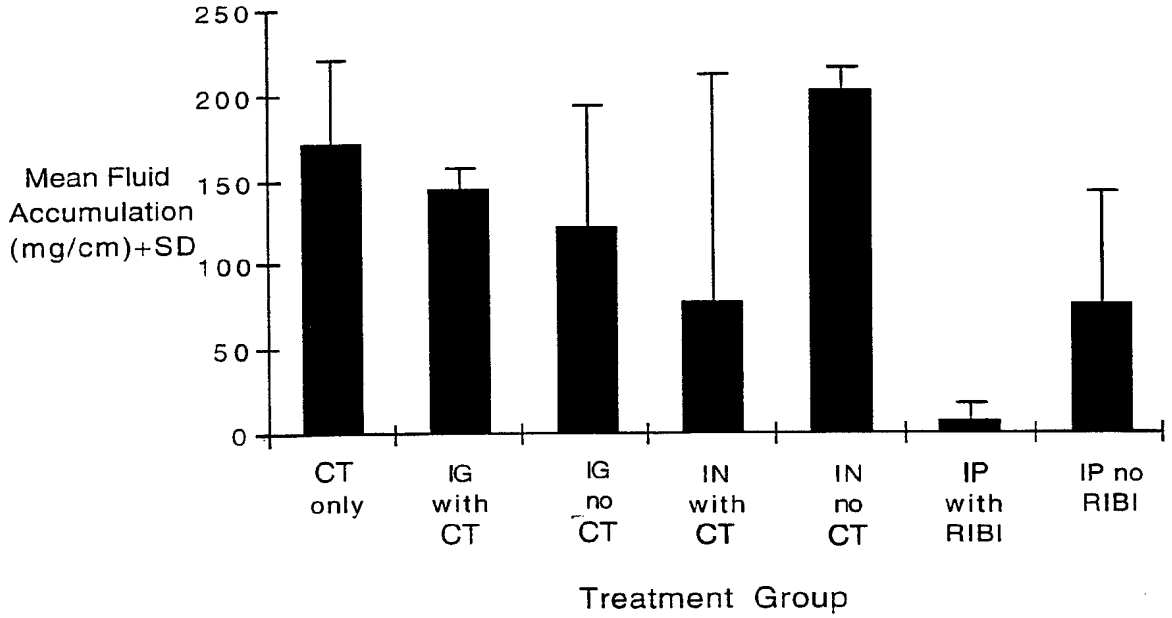


FIG. 13B

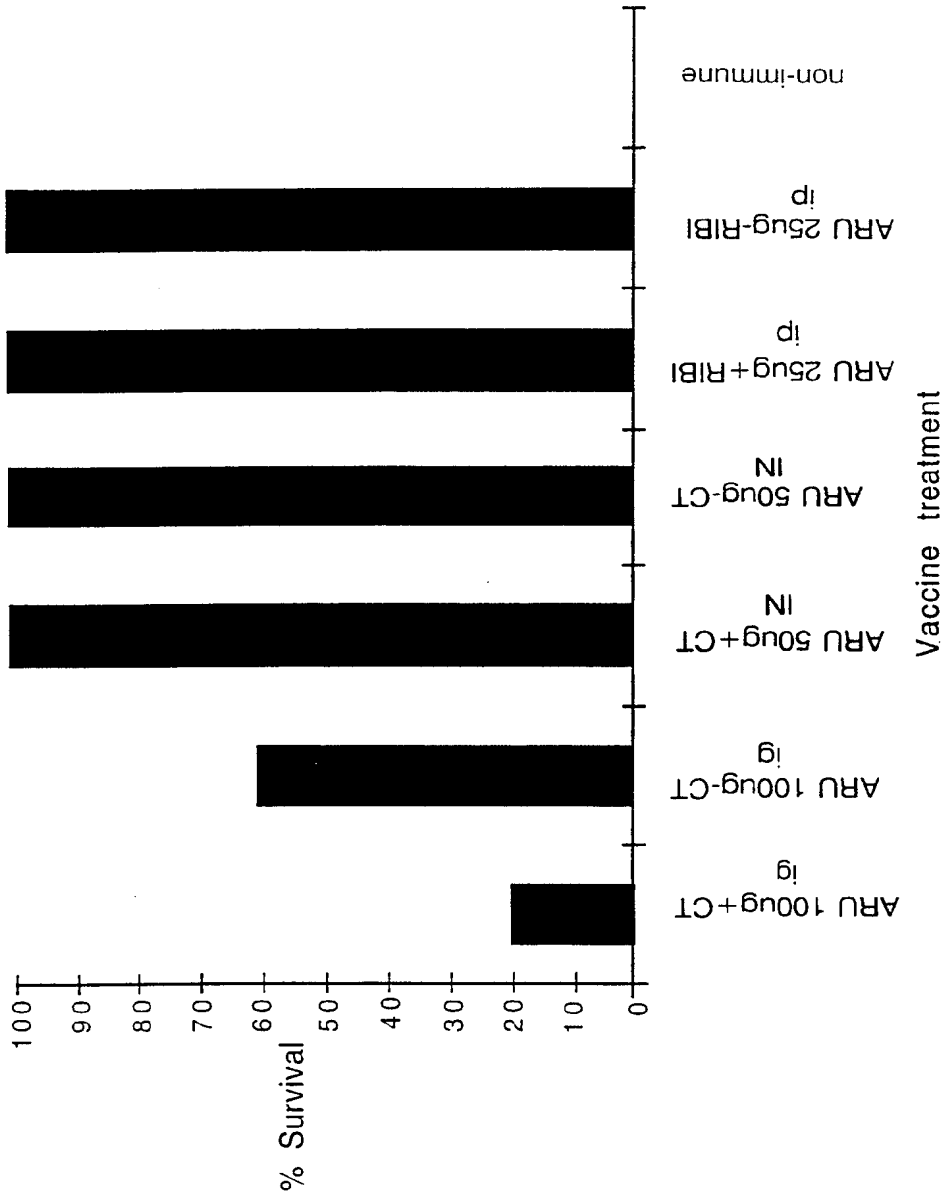

% Survival
100
90
80
70
60
50
40
30
20
10
0
ARU 100ug+CT ig
ARU 100ug-CT ig
ARU 50ug+CT IN
ARU 50ug-CT IN
ARU 25ug+RIBI ip
ARU 25ug-RIBI ip
non-immune
Vaccine treatment

FIG. 14

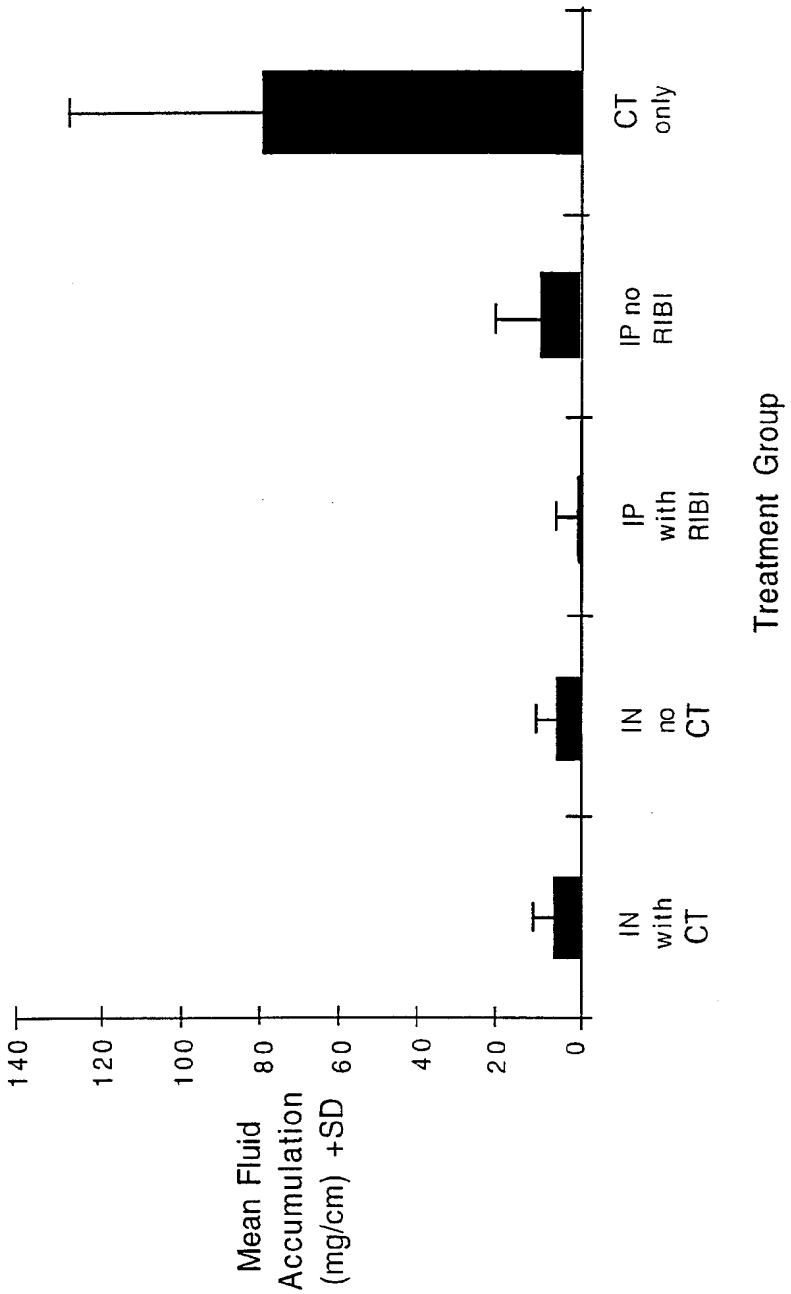

Mean Fluid Accumulation (mg/cm) +SD
140
120
100
80
60
40
20
0
IN with CT
IN no CT
IP with RIBI
IP no RIBI
CT only
Treatment Group

FIG. 15

INTERNATIONAL SEARCH REPORT

Inter...onal application No.
PCT/US96/10987

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/00, 39/02, 39/08, 38/00; A01N 37/18; C07K 2/00
US CL :424/184.1, 192.1, 236.1, 239.1, 247.1; 514/2; 530/300, 825
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 192.1, 236.1, 239.1, 247.1; 514/2; 530/300, 825

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, MEDLINE, EMBASE, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 9413264 A1 (OPHIDLAN PHARMACEUTICALS, INC.) 23 June 1994, see entire document.	1-15
Y, P	TORRES, et al. Evaluation of Fromalin-Inactivated Clostridium difficile Vaccines Administered by Parenteral and Mucosal Routes of Immunization in Hamsters. Infection and Immunity. December 1995, Vol. 63, No.12, pages 4619-4627, see entire document.	1-15
Y	PAPPO, et al. Effect of Oral Immunization with Recombinant Urease on Murine Helicobacter felis Gastritis. Infection and Immunity. April 1995, Vol. 63, No. 4, pages 1246-1252, see entire document.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 AUGUST 1996	Date of mailing of the international search report 31 OCT 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Khalid Masood for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10987

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOLMGREN, et al. Strategies for the Induction of Immune Responses at Mucosal Surfaces Making Use of Cholera Toxin B Subunit as Immunogen, Carrier, and Adjuvant. Am. J. Trop. Med. Hyg. 1994, Vol. 50, No. 5 Suppl, pages 42-54, see entire document.	1-15
Y	MICHETTI, et al. Immunization of BALB/c Mice Against Helicobacter felis Infection With Helicobacter pylori Urease. Gastroenterology. 1994, Vol. 107, pages 1002-1011, see entire dcoument.	1-15
Y	LEE, et al. Oral Immunization with Recombinant Helicobacter pylori Urease Induces Secretory IgA Antibodies and Protects Mice from Challenge with Helicobacter felis. The Journal of Infectious Diseases. 1995, Vol. 172, pages 161-172, see entire document.	1-15
A	US 5,182,109 A (TAMURA et al.) 26 January 1993, see entire document.	1-15
A	BARROSO et al. Mutagenesis of the Clostridium difficile toxin B gene and effect on cytotoxic activity. Microbial Pathogenesis. 1994, Vol. 16, pages 297-303, see entire document.	1-15
A	US 4,944,942 A (BROWN ET AL) 31 July 1990, see entire document.	1-15